Agilent

AssayMAP Protein Sample Prep Workbench

User Guide

Original Instructions

Notices

Manual Part Number

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WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

Contents

Preface

This preface contains the following topics:

About this guide

Overview

This guide includes the user guides for the applications and utilities in the AssayMAP Protein Sample Prep Workbench.

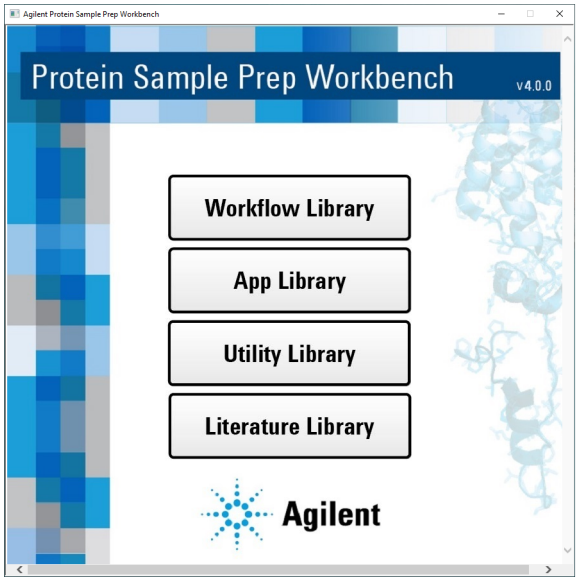
Software version

This guide documents the following software versions:

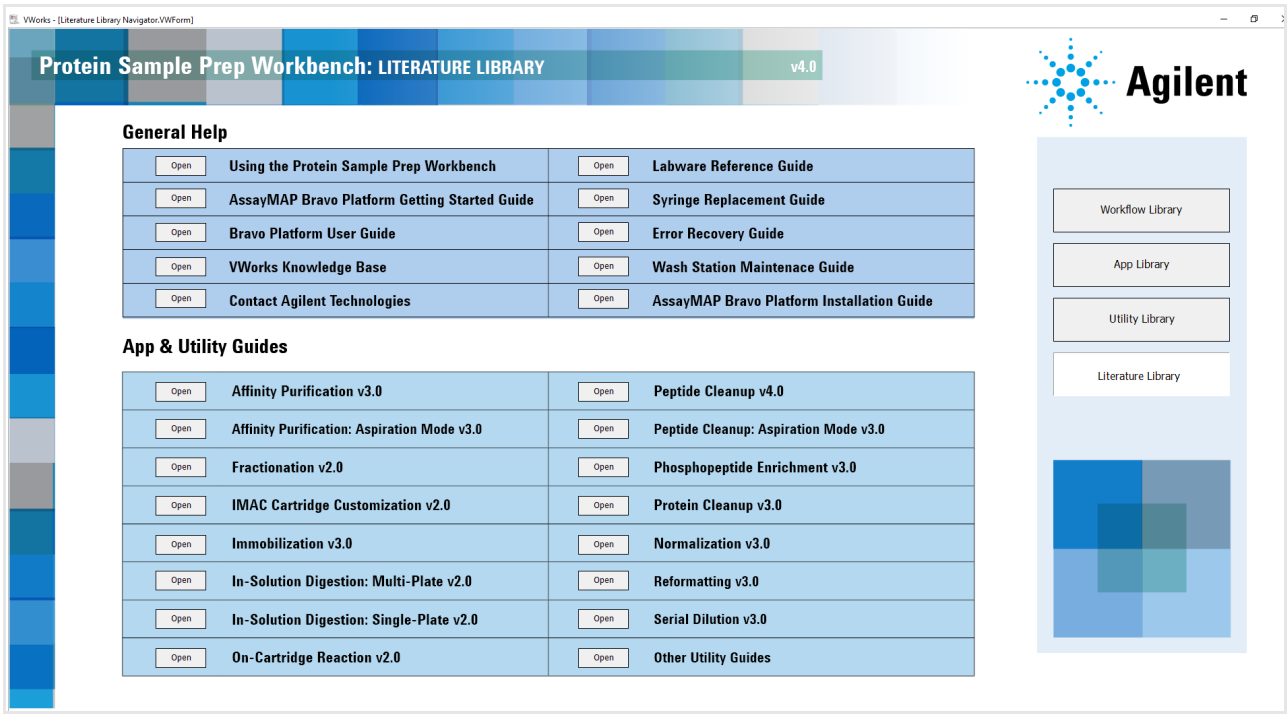
- Protein Sample Prep Workbench 4.0
- VWorks Automation Control 14.1.1

Accessing the user guides

You can find the user guides for the AssayMAP applications and utilities in the Protein Sample Prep Workbench.



The Literature Library provides links to the user guides.



The app quick start guides are available in the App Library and additional utility guides are available in the Utilities Library.

Reporting problems

If you find a problem with the AssayMAP Bravo Platform, contact Agilent Automation Solutions Technical Support. For contact information, go to https://www.agilent.com/en/contact-us/page.

To report problems with...	Have the following information ready
Hardware	• Instrument serial number from the Bravo serial number label • Short description of the problem
Software	• Instrument serial number from the Bravo serial number label • Short description of the problem • Relevant software version number (for example, automation control software, diagnostics software, and firmware) • Error message text (or screen capture of the error message dialog box) • Relevant files, such as log files
AssayMAP Cartridges	• Cartridge type • Lot number • Short description of the problem

For instructions on how to resolve common error messages, see the *AssayMAP Bravo Platform Error Recovery Guide*.

1 Getting started

This section contains the following topics:

Starting the workbench and logging in

For instructions on how to start up the AssayMAP Bravo Platform, see "System Startup/Shutdown v3.0 User Guide" on page 574.

Starting the Protein Sample Prep Workbench

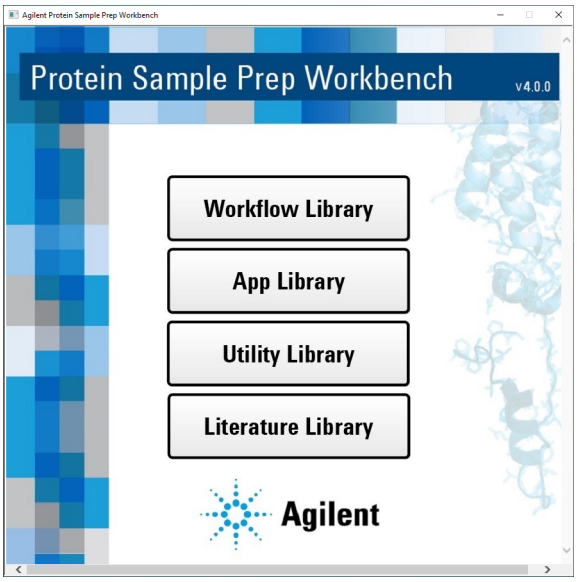
VWorks administrator, technician, operator, or guest privileges are required to log in to the software. Contact your administrator if you need login credentials.

To start the Protein Sample Prep Workbench:

1 Double-click 

on the Windows desktop.

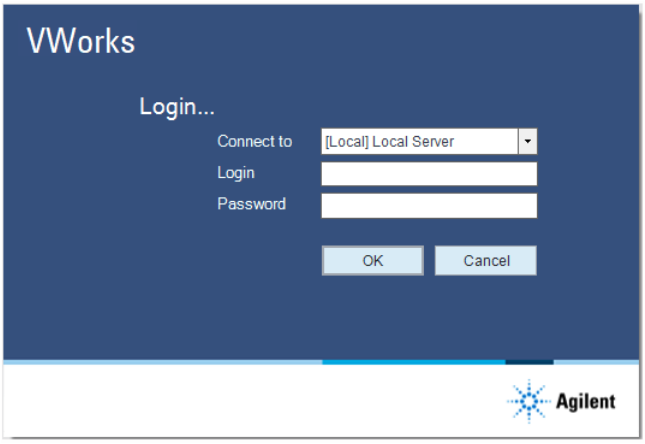
The Protein Sample Prep Workbench window opens.



2 Click one of the following buttons in the Workbench window:

- Workflow Library
- Application Library
- Utility Library
- Literature Library

The VWorks software starts and the VWorks Login window opens.



3 In the **VWorks Login** window, type your **Login** and **Password**, and then click **OK.**

The VWorks window opens. After a few seconds, the selected Library page opens in the VWorks window. For a description of each Library, see "About the Protein Sample Prep Workbench" on page 4.

Changing your password

The following procedure is applicable if Open Lab Control Panel is configured to use Internal authentication. If Open Lab Control Panel is configured to use Windows Domain, see the Windows documentation for instructions on how to change your password.

If you need help changing your password, contact your administrator.

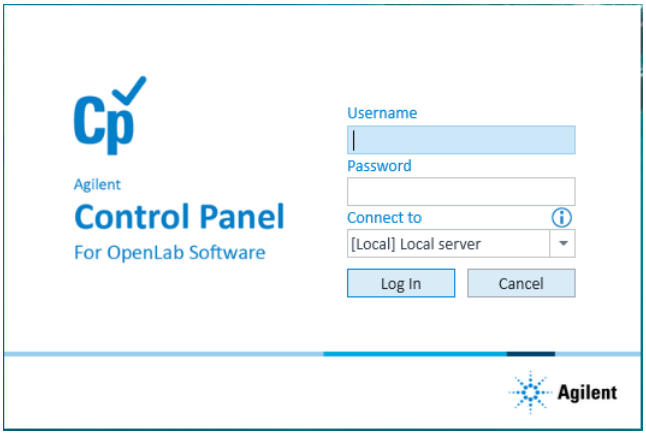
Changing your password in the OpenLab Control Panel

To log in to Control Panel and change your VWorks user password:

1 Double-click  on the Windows desktop to start OpenLab Control Panel.

Alternatively, select **Start ( )** > **All Apps** > **Agilent Technologies** > **Control Panel.**

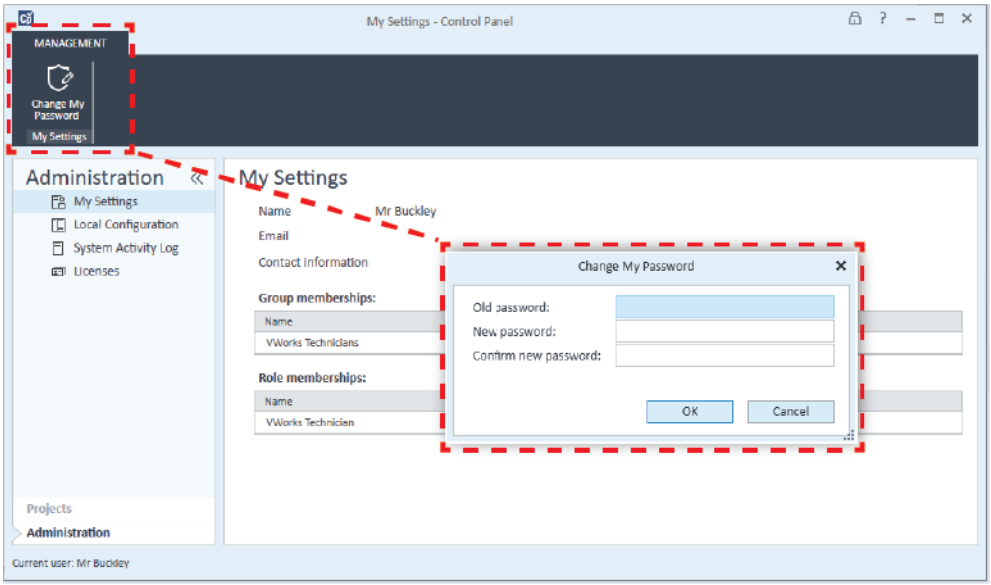
2 In the Control Panel login window, enter your user name and password, and click **Log In**.



If you log in as a VWorks technician, operator, or guest, the Control Panel opens to the My Settings page.

3 In the ribbon at the top of the **My Settings** page, click **Change My Password**.

4 In the **Change My Password** dialog box, type the **Old password**, type the new password in the **New password** and **Confirm new password** boxes, and then click **OK**.



5 If you have finished viewing the My Settings page, close the Control Panel.

About the Protein Sample Prep Workbench

The Protein Sample Prep Workbench includes the following:

- Workflow Library. For details, see "About the Workflow Library" on page 5.
- Application Library. For details, see "About the App Library" on page 7.
- Utility Library. For details, see "About the Utility Library" on page 9.
- Literature Library. For details, see "About the Literature Library" on page 10.

Major and minor revisions of apps and utilities

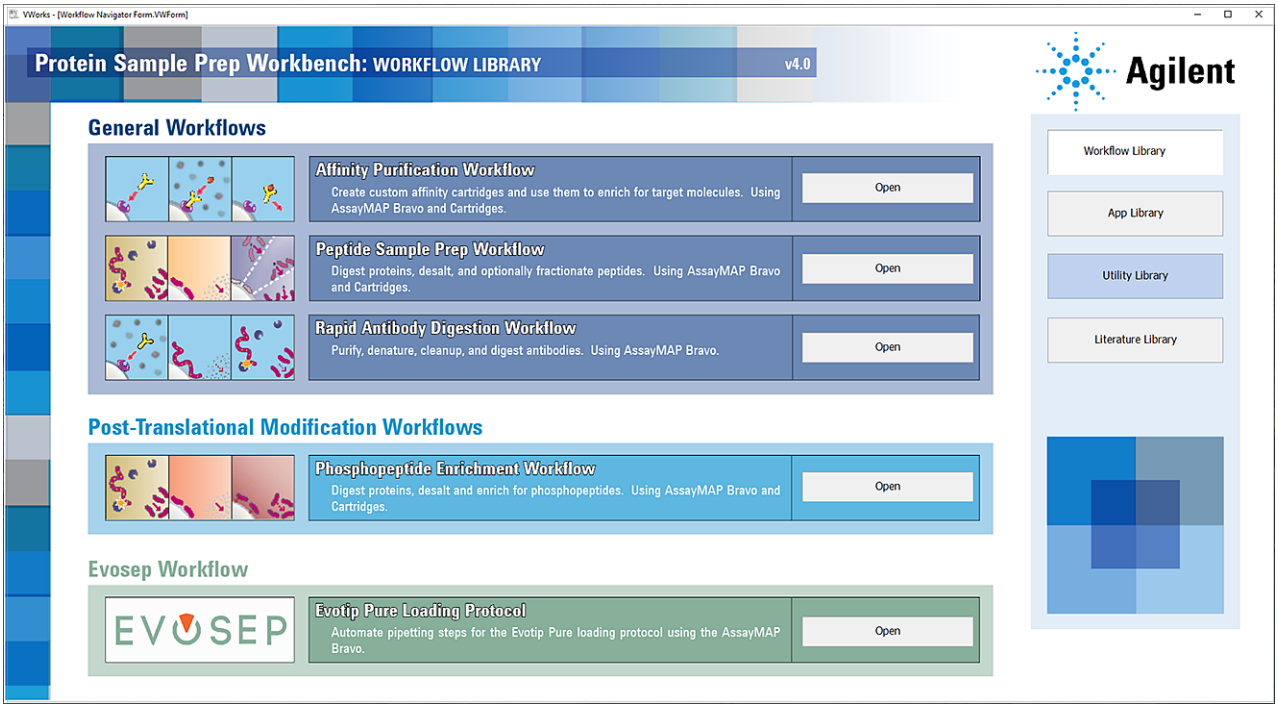
The version of the Protein Sample Prep Workbench, each application, and each utility is designated by a decimal number, where

- Major revision or original release is designated by the number before the decimal point. A major revision is a change to the protocol and may also include changes to the interface (AssayMAP form).
- Minor revision is designated by the number after the decimal point. Minor revisions indicate a change only to the AssayMAP form and does not impact how the protocol runs.

For example, version 1.0 designates an original release of the application or utility, version 1.1 designates a change to the form only, version 2.0 designates a change to the protocol, and so forth. A major version automatically resets the minor number to 0.

About the Workflow Library

The Workflow Library is a collection of protein sample preparation workflows. Each workflow consists of a set of applications that are performed in sequence to complete an experiment. In addition, the workbench contains links to utilities that facilitate sample and reagent preparation, the transition between applications, and the transition between the output of the final application and the input of the analytical instrument.



General Workflows

Workflow	Enables the …
Affinity Purification	Creation of custom affinity cartridges that can be used to enrich target molecules. The workflow consists of the following applications: • Immobilization. For details, see the "Immobilization v3.0 User Guide" on page 193. • Affinity Purification. For details, see the "Affinity Purification v3.0 User Guide" on page 45.
Peptide Sample Prep	Digestion of proteins, the desalting of the peptide resulting from the digestion, and optionally the fractionation of the peptide mixture. The workflow consists of the following applications: • In-Solution Digestion. For details, see the "In-Solution Digestion: Single Plate v2.0 User Guide" on page 275. • Peptide Cleanup. For details, see the "Peptide Cleanup v4.0 User Guide" on page 355. • Fractionation. For details, see the "Fractionation v2.0 User Guide" on page 125.
Rapid Antibody Digestion	Desalting of denatured antibodies, and the rapid digestion of these antibodies. The workflow consists of the following applications: • Affinity Purification. For details, see the "Affinity Purification v3.0 User Guide" on page 45. • In-Solution Digestion. For details, see the"In-Solution Digestion: Single Plate v2.0 User Guide" on page 275. • Protein Cleanup. For details, see the "Protein Cleanup v3.0 User Guide" on page 467.

Post-Translational Modification Workflows

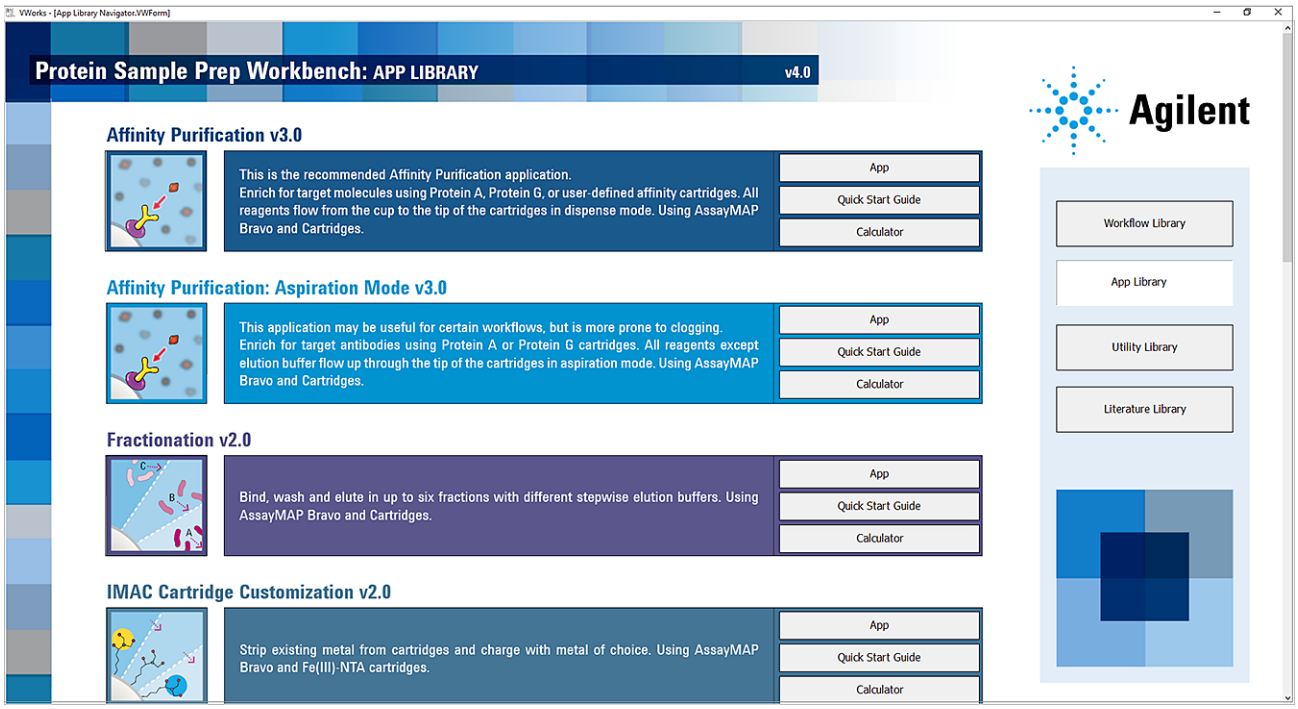
Workflow	Enables the …
Phosphopeptide Enrichment	Digestion of proteins, the desalting of the peptide resulting from the digestion, and the enrichment of the phosphopeptides. The workflow consists of the following applications: • In-Solution Digestion. For details, see the In-Solution Digestion. For details, see the "In-Solution Digestion: Single Plate v2.0 User Guide" on page 275. • Peptide Cleanup. For details, see the "Peptide Cleanup v4.0 User Guide" on page 355. • Phosphopeptide Enrichment. For details, see the "Phosphopeptide Enrichment v3.0 User Guide" on page 425.

Evosep Workflow

Workflow	Enables the …
Evosep 96AM Workflow	Automation of the pipetting steps for the Evotip Pure loading protocol. For details, see the *Evosep - 96AM Workflow Quick Start Guide* at "Quick start guides" on page 1.

About the App Library

The App Library contains a collection of ready-to-use sample processing applications that can be used individually or in various combinations to address your experimental needs.



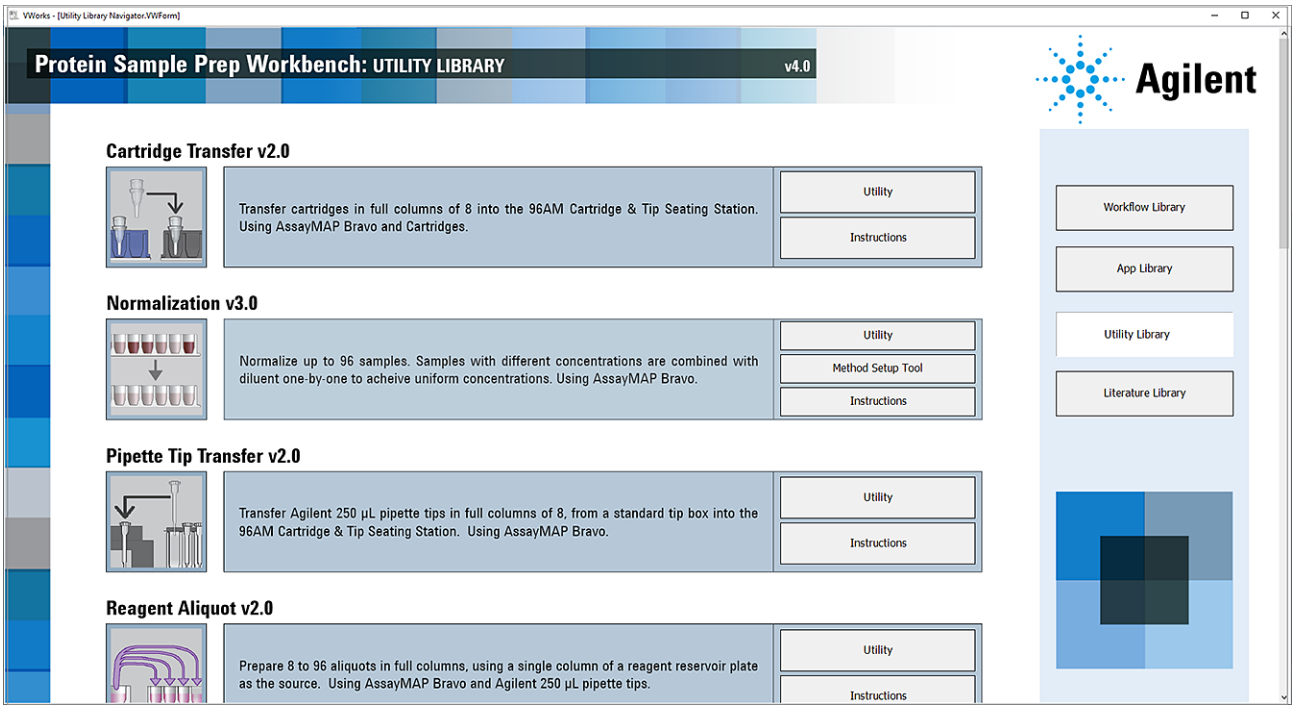
Available applications

Application	Automates the …	See…
Affinity Purification v3.0	Affinity purification of target molecules using AssayMAP cartridges. This is the recommended Affinity Purification application.	"Affinity Purification v3.0 User Guide" on page 45

Application	Automates the …	See…
Affinity Purification: Aspiration Mode v3.0	Affinity purification of target molecules using AssayMAP cartridges. The sample load and wash steps in this protocol are drawn into rather than pushed through the cartridges, which is in contrast to the Affinity Purification v3.0 protocol. This application may be useful for certain workflows, but is more prone to clogging.	"Affinity Purification: Aspiration Mode v3.0 User Guide" on page 89
Fractionation v2.0	Stepwise fractionation of samples from AssayMAP cartridges in up to six fractions using buffers with increasing ionic strength, increasing pH, or increasing organic concentration.	"Fractionation v2.0 User Guide" on page 125
IMAC Cartridge Customization v2.0	Stripping and charging of AssayMAP Fe(III)-NTA cartridges with the desired metal of choice for immobilized metal affinity chromatography (IMAC) experiments.	"IMAC Cartridge Customization v2.0 User Guide" on page 159
Immobilization v3.0	Immobilization of antibodies and other ligands to PAW, PGW, or SAW cartridges.	"Immobilization v3.0 User Guide" on page 193
In-Solution Digestion: Single Plate v2.0	Digestion of 8 to 96 protein samples in a 96-well microplate. The protocol includes five generic liquid-handling steps that successively transfer a reagent from a reagent plate at deck location 5, 6, 7, 8, or 9, into the Sample Plate at deck location 4.	"In-Solution Digestion: Single Plate v2.0 User Guide" on page 275
In-Solution Digestion: Multi-Plate v2.0	Digestion of 8 to 384 protein samples in up to four 96-well microplates in a single run.	"In-Solution Digestion: Multi-Plate v2.0 User Guide" on page 235
On-Cartridge Reaction v2.0	Reactions on AssayMAP cartridges that contain immobilized target molecules.	"On-Cartridge Reaction v2.0 User Guide" on page 309
Peptide Cleanup v4.0	Cleanup of peptide samples using AssayMAP cartridges. This is the recommended Peptide Cleanup application.	"Peptide Cleanup v4.0 User Guide" on page 355
Peptide Cleanup: Aspiration Mode v3.0	Cleanup of peptide samples using AssayMAP cartridges. The sample load and wash steps in this protocol are drawn into rather than pushed through the cartridges, which is in contrast to the Peptide Cleanup v4.0 protocol. This application may be useful for certain workflows, but is more prone to clogging.	"Peptide Cleanup: Aspiration Mode v3.0 User Guide" on page 393
Phosphopeptide Enrichment v3.0	Phosphopeptide enrichment using the AssayMAP Fe(III)-NTA or TiO_2 cartridges.	"Phosphopeptide Enrichment v3.0 User Guide" on page 425
Protein Cleanup v3.0	Cleanup of protein samples using the AssayMAP RP-W cartridges.	"Protein Cleanup v3.0 User Guide" on page 467

About the Utility Library

The Utility Library is a collection of protocols that automate specific tasks (for example, liquid handling) to prepare the system for an application protocol, transition between applications in a workflow, or transition between an application and loading onto an analytical device. For example, the System Startup/Shutdown utility initializes the AssayMAP Bravo Platform, washes the syringes, and primes the wash lines and the wash station.

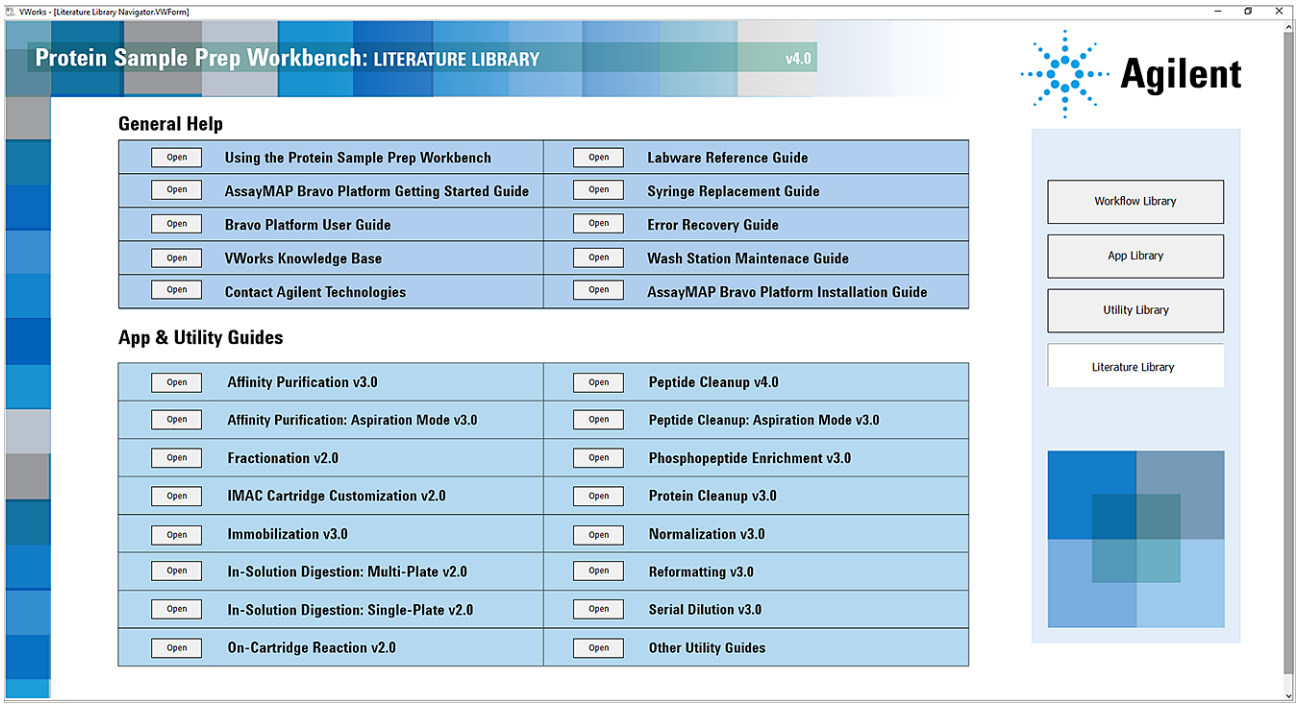


Utility	Description	See...
Cartridge Transfer v2.0	Moves full columns of AssayMAP cartridges from the source cartridge rack to the 96AM Cartridge & Tip Seating Station.	"Cartridge Transfer v2.0 User Guide" on page 506
Normalization v3.0	Automates the normalization of up to 96 samples in microplate format.	"Normalization v3.0 User Guide" on page 585
Pipette Tip Transfer v2.0	Moves full columns of disposable pipette tips from the source tip box to the 96AM Cartridge & Tip Seating Station.	"Pipette Tip Transfer v2.0 User Guide" on page 512
Reagent Aliquot v2.0	Aliquots a reagent from a single bulk reagent reservoir into 1–12 columns of a microplate or reservoir. The utility uses a single column of eight Agilent 250-µL pipette tips to prepare the aliquots.	"Reagent Aliquot v2.0 User Guide" on page 518

Utility	Description	See...
Reagent Transfer v3.0	Transfers 1–12 columns of reagents in parallel from a reagent source plate or reservoir into a 96-well plate.	"Reagent Transfer v3.0 User Guide" on page 525
Reformatting v3.0	Transfers solutions from any well in one microplate to any well in another microplate.	"Reformatting v3.0 User Guide" on page 623
Serial Dilution v3.0	Creates a Serial Dilution plate with up to 24 dilutions and up to 5 replicates.	"Serial Dilution v3.0 User Guide" on page 653
Single Liquid Addition v2.0	Adjusts the buffer composition or adds a component to up to four sample plates.	"Single Liquid Addition v2.0 User Guide" on page 542
Syringe Test v2.0	Verifies the integrity of the probes and syringes in the Bravo 96AM Head.	"Syringe Test v2.0 User Guide" on page 549
Syringe Wash v3.0	Washes the syringes after an application or utility run with the solution of your choice for the specified number of wash cycles to avoid run to run cross-contamination.	"Syringe Wash v3.0 User Guide" on page 567
System Startup v3.0	Initializes the AssayMAP Bravo Platform, washes the syringes, and primes the wash station and wash lines.	"System Startup/Shutdown v3.0 User Guide" on page 574
System Shutdown v3.0	Prepares the AssayMAP Bravo Platform for idle time by washing the syringes and then aspirating 200 µL of Syringe Storage Liquid into the syringes.	"System Startup/Shutdown v3.0 User Guide" on page 574

About the Literature Library

The Literature Library provides links to the user documentation for the AssayMAP Bravo Platform.



General Help section of Literature Library

Title	Description
Using the Protein Sample Prep Workbench	Provides an overview of the workbench features.
AssayMAP Bravo Platform Getting Started Guide	Provides an overview of the platform and a detailed description of the AssayMAP liquid-handling head.
Bravo Platform User Guide	Explains how to set up and operate the Bravo Platform and how to configure accessories.
Labware Reference Guide	Provides detailed information and a photo of each labware option for the workbench applications.
Syringe Replacement Guide	Provides the procedures for replacing damaged syringes in the AssayMAP head.
Error Recovery Guide	Provides guidelines for how to recover from the more common error messages that may occur when using the AssayMAP Bravo Platform.
Wash Station Maintenance Guide	Provides the procedures for inspecting the chimneys and replacing damaged chimneys.
AssayMAP Bravo Platform Installation Guide	Explains how to install the AssayMAP Bravo Platform, including configuring the deck accessories and setting the teachpoints.

Title	Description
VWorks Knowledge Base	Provides the help system for the VWorks software, including: • Configuration and administration guides and quick references for VWorks Networked, VWorks Plus, and VWorks Standard editions • VWorks Setup Guide • VWorks User Guide • Bravo safety, installation, user, and quick-start guides • User guides for other VWorks-controlled devices

App & Utility Guides section of Literature Library

The Protein Sample Prep Workbench user guides describe how to prepare and run protocols for various sample prep applications and utilities. The topics are for both routine operators and assay developers.

Compliance-enabling features

The combination of features in VWorks Plus 14.1.1.1 and Protein Sample Prep Workbench 4.0 help enable compliance with Part 11 of Title 21 of the Code of Federal Regulations (21 CFR Part 11). The FDA rules and guidelines for compliant electronic records and computerized systems require secure data handling, including:

- *Data security*. Physical protection of data by limiting access to the system and preventing unauthorized access.
- *Data integrity*. Protecting raw data and metadata and preventing these from unauthorized modification, and linking raw data and results to reproduce the original results at any time, for example, in an audit situation, and document each new result copy.
- *Audit traceability*. Documenting who did what to the results and when.

VWorks Plus features that support compliance

VWorks Plus compliance-enabling features include user authentication, content management, tamper detection, audit trails, electronic signatures, and activity logs. For a description of these features, see the Using compliance features chapter in the *VWorks Automation Control Setup Guide*.

Workbench features that support compliance

The Protein Sample Prep Workbench uses experiment IDs and methods to support traceability and data integrity. An experiment ID and a method are required to run any application or utility in the compliance-enabled Protein Sample Prep Workbench.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  Electronic signatures are required to close an experiment ID. The software automatically generates a report when an experiment ID is closed. A report describes who did what and when for a given experiment ID.

  For details, see "Using Experiment IDs" on page 23.
- A *method* is a comprehensive collection of saved settings for an application or utility that you can use to run the application or utility.

  Methods in the OpenLab Content Management storage can be used to run an app or utility protocol.

  Any changes to an existing method must be saved using a new name before the revised method can be used to run an app or utility protocol.

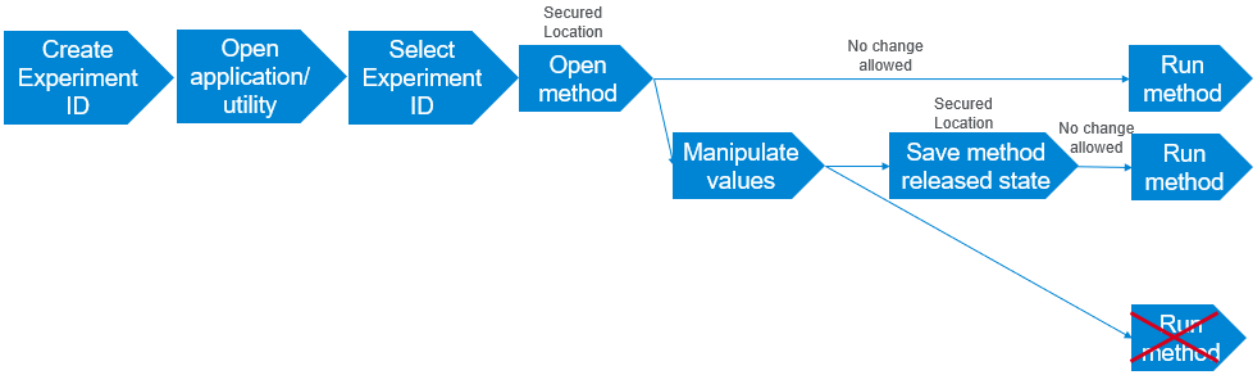
  For detailed procedures, see the user guide for the app or utility.

Roles and privileges

The VWorks administrator, technician, and operator roles have the following privileges in the compliance-enabled Workbench.

VWorks role	Privileges for experiment IDs and methods
VWorks operator	Experiment IDs • Select an experiment ID to run an app or utility • Add notes to an experiment ID • Generate an experiment ID report • Export experiment IDs Methods • Select a method to run an app or utility • Export a method
VWorks technician	All the privileges of a VWorks operator, plus the following: Experiment IDs • Create experiment IDs and edit the description of experiment IDs that have the Not Yet Used status • Close experiment IDs and post a signature when closing • Import experiment IDs Methods • Create and save methods • Import methods **IMPORTANT** Any new or modified method must be saved using a unique name before it can be run, as the following figure shows.
VWorks administrator	All the privileges of a VWorks operator and technician, plus the following: • Archive closed experiment IDs • Delete experiment IDs that have a Not Yet Used status • Log in to Content Browser and edit the project, including deleting files

Figure VWorks Technician privileges for creating and running methods



Overview of software architecture

The AssayMAP Bravo Platform requires the following software, which runs on a single computer workstation:

- Agilent Protein Sample Prep Workbench. A collection of simple form-based user interfaces for running the applications and utilities that control the AssayMAP Bravo Platform. Each application and utility has default methods that can be altered and saved. The Protein Sample Prep Workbench runs within the VWorks software.
- Microsoft Excel. Required for the Workbench method setup tools, reagent volume calculators, and Syringe Test data analysis tool.
- Agilent VWorks software 14.1.1. A combination of Agilent OpenLab software components and Agilent VWorks software that provides the user management and instrument control for the AssayMAP Bravo Platform. The software is available in the following editions.

VWorks edition	File storage type	Compliance enabled?
VWorks Plus	Content Management *Note:* A system or VWorks administrator can use the Content Browser to view and edit the project structure and contents at /VWorks Projects/VWorks/	Yes
VWorks Standard	Local file system *Note:* Any user can access the project files directly using the Windows File Explorer at C:\OLSS Projects\VWorks Projects\VWorks\	No

The following figures show the software architecture for each VWorks edition. As the figures show, the OpenLab components include a Control Panel, which is the user interface for the Shared Services. The Shared Services, such as user access, software licenses, and storage are configured initially when the software is installed. For more details on these OpenLab components, see the *VWorks Plus Configuration and Administration Guide* or *VWorks Standard Configuration and Administration Guide*.

Figure Architecture of VWorks Plus and Protein Sample Prep Workbench

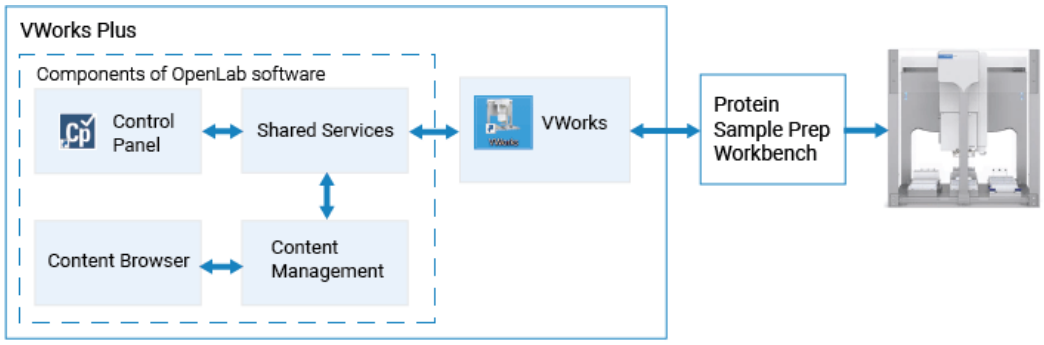
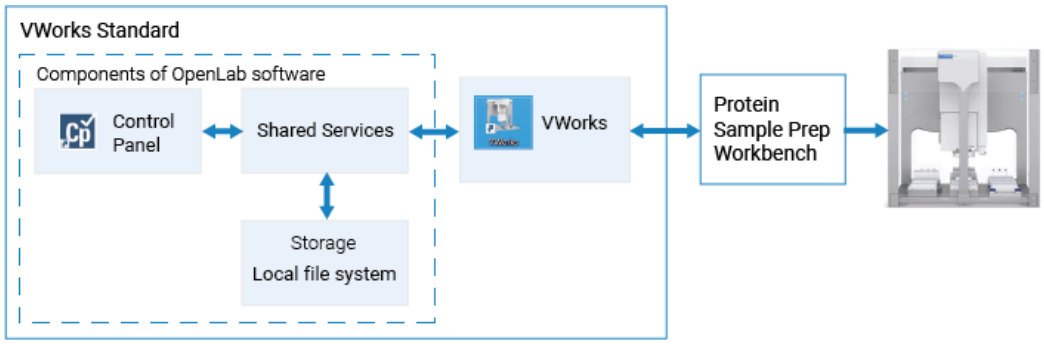


Figure Architecture of VWorks Standard and Protein Sample Prep Workbench



The VWorks files, such as device files, profiles, labware, protocols, and the like are stored as .roiZip archives. All file modifications must be handled within the VWorks software.

IMPORTANT The VWorks software cannot reload files (.roiZip extension) that have been modified or renamed outside of the VWorks software.

Exporting and importing AssayMAP methods

A *method* is a comprehensive collection of saved settings that you can use to run a Workbench application or utility. Each application and utility in Protein Sample Prep Workbench 4.0 has default methods that you can use. Each app and utility user guide describes how to select and how to create methods.

The VWorks export and import features enable the sharing of customized AssayMAP methods with users on a different computer. This topic describes how to export and import AssayMAP methods, one method at a time.

Prerequisites

Both computers must be running the following software:

- VWorks software 14.1.1
- Protein Sample Prep Workbench 4.0

VWorks technician privileges or greater are required to import AssayMAP method files into the Shared Services storage. No overwrite is allowed for AssayMAP method files that already exist in Shared Services storage.

The VWorks menu bar must be visible to access the export and import commands.

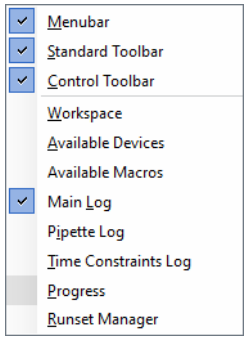
Displaying the VWorks Tool menu

To display the VWorks main window, do one of the following:

- If the Protein Sample Prep Workbench is not currently running, start the VWorks software and log in when prompted to do so.

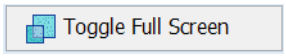
  

  If the menubar is not visible in the VWorks main window, right-click the window and select **Menubar** in the shortcut menu that appears.

- If the Protein Sample Prep Workbench is already running, use the following procedure to turn off Full Screen mode so that the VWorks File menu is visible.

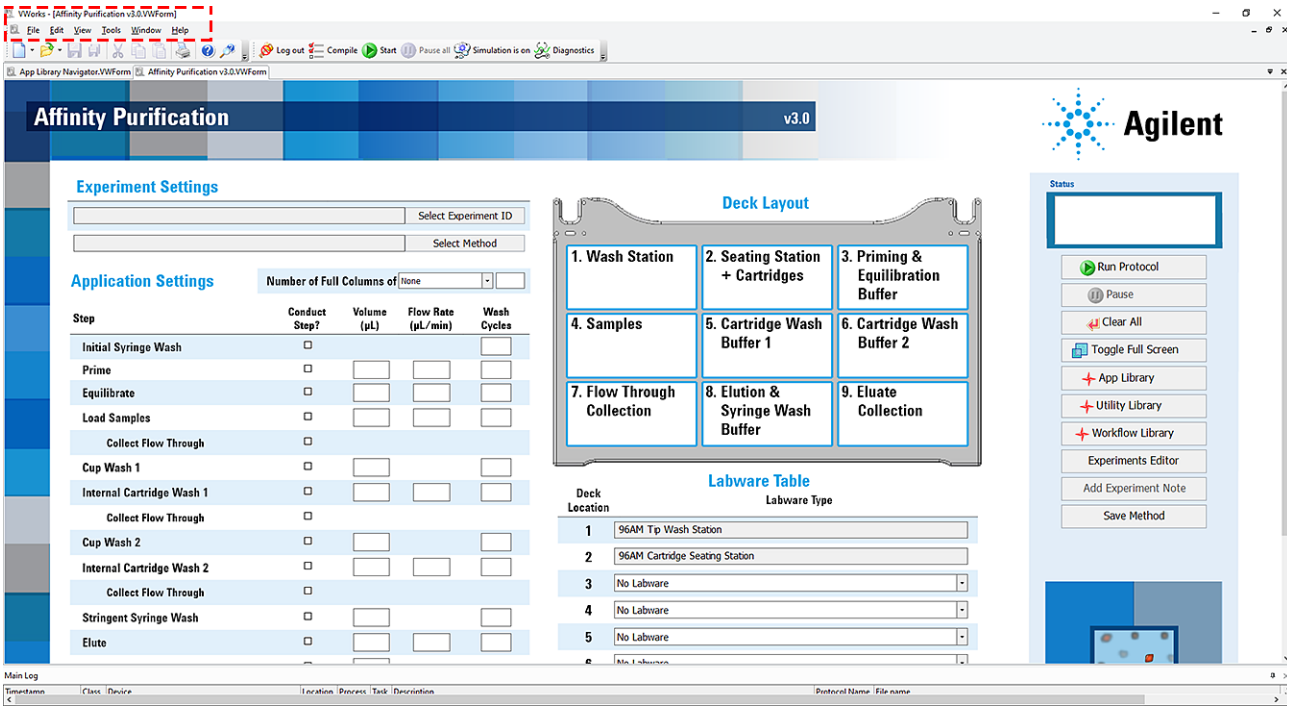
To turn off Full Screen mode:

1 In any app or utility, click 

in the navigation pane of the form.

2 Ensure that the VWorks menu bar is visible above the Workbench form, as the following figure shows.

Figure Example of VWorks Menubar visible in Full Screen off mode



AssayMAP method file storage

The AssayMAP method files are stored in the following Shared Services locations.

VWorks edition	Storage location
VWorks Plus	/VWorks Projects/VWorks/AM Methods/
VWorks Standard	C:/OLSS Projects/VWorks Projects/VWorks/AM Methods/

The AM Methods folder stores the method files (.mth) for each app and utility in a corresponding subfolder of the same name. The file names for the default settings are the same for multiple apps and utilities. However, only the app or utility used to create a method can open that method.

For example,

- AM Affinity Purification 3.0 subfolder contains the Affinity Purification methods:
  - 25uL Cartridge Default Settings.mth
  - 5uL Cartridge Default Settings.mth
- AM Peptide Cleanup 4.0 subfolder contains the Peptide Cleanup methods:
  - 25uL Cartridge Default Settings.mth
  - 5uL Cartridge Default Settings.mth
- AM Reagent Transfer v3.0 subfolder contains the Reagent Transfer method:

  Default Settings.mth

- AM Syringe Wash v3.0 subfolder contains the Syringe Wash method:
  Default Settings.mth

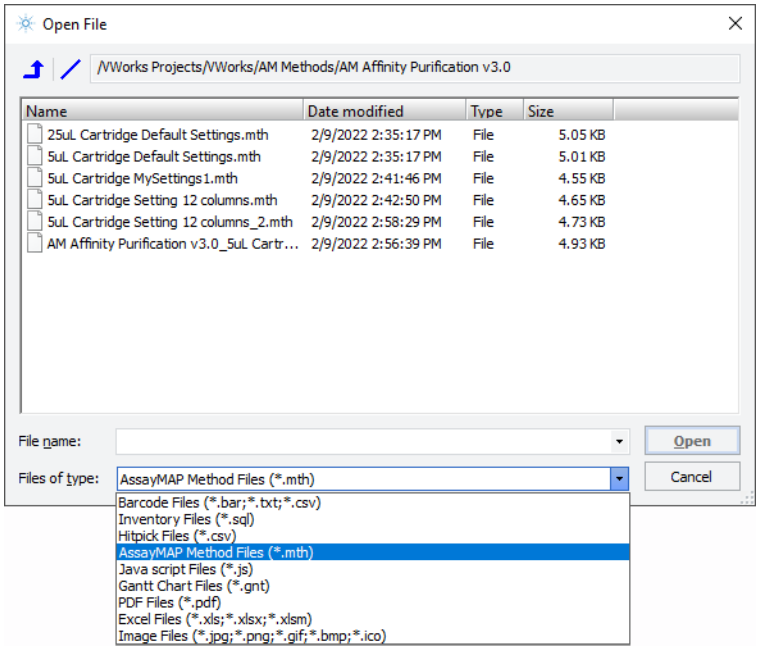
To help prevent accidentally importing a method file into the wrong subfolder, the software prepends the corresponding app or utility folder name to the file name when you export a method.

IMPORTANT Agilent recommends that you retain the exported file name assigned by the software until that file is imported to the correct subfolder. If desired, you can remove the folder name from a file name when you import it, after navigating to the correct subfolder with the same folder name.

Exporting an AssayMAP method

To export an AssayMAP method file (.mth):

1 In the VWorks window, click **File > Export Misc File**. The Open File dialog box appears.

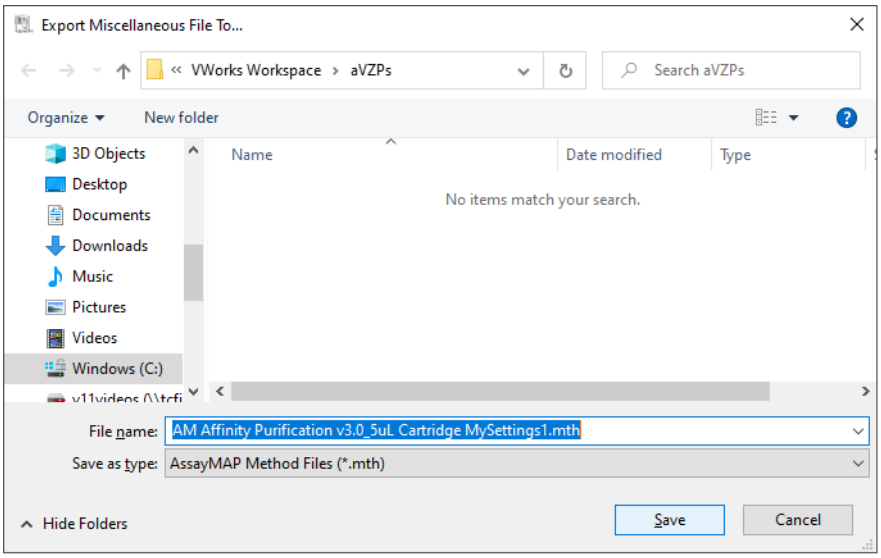


2 In the **Open File** dialog box:

a Click **AssayMAP Method Files (*.mth)** in the **Files of type** list.

b Use the button to navigate to the AM Methods app or utility subfolder.

c Select the method file to be exported, and then click **Open**.
The **Export Miscellaneous File To** dialog box opens.



3 In the **Export Miscellaneous File To** dialog box:

IMPORTANT

Agilent recommends retaining the folder name as part of the exported file name to help ensure that the file is imported to the correct method subfolder for the corresponding app or utility.

a Notice that the proposed **File name** includes the name of the app or utility subfolder of the method file you are exporting.

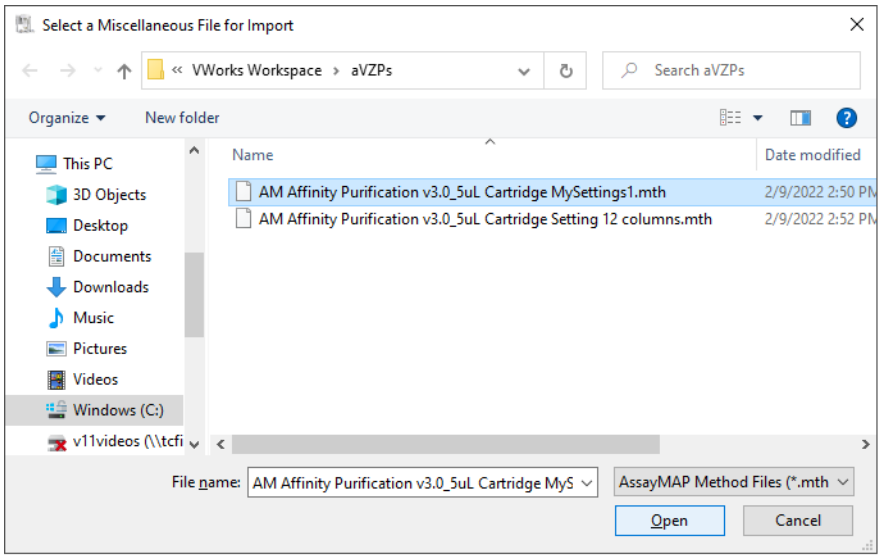
b Ensure that the **Save as type** is **AssayMAP Method Files (*.mth)**.

c Navigate to the export location, and then click **Save**.

Importing an AssayMAP method

To import an AssayMAP method file (.mth):

1 In the VWorks window, click **File > Import Misc File**. The Select a Miscellaneous File for Import dialog box opens.

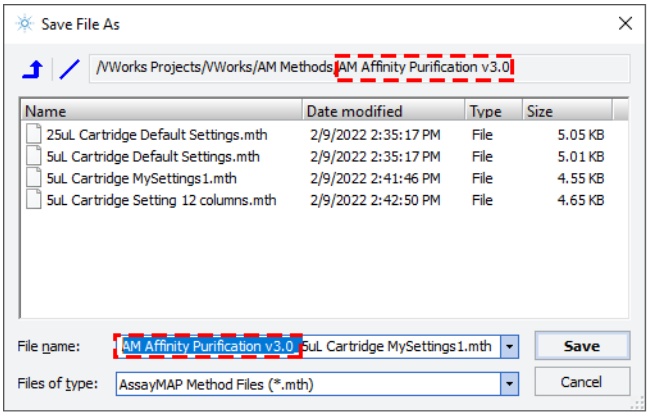


2 In the Select a Miscellaneous File for Import dialog box:

   a Ensure that the **AssayMAP Method Files (*.mth)** file type is selected.

   b Navigate to the file location, select the method file to be imported, and then click **Open**.

3 In the **Save File As** dialog box that opens, do the following:



   a Use the button to navigate to the corresponding AM Methods app or utility subfolder.

   Ensure that you select the subfolder for the app or utility that created this method.

   b Ensure the file name is different from the other files in the folder.

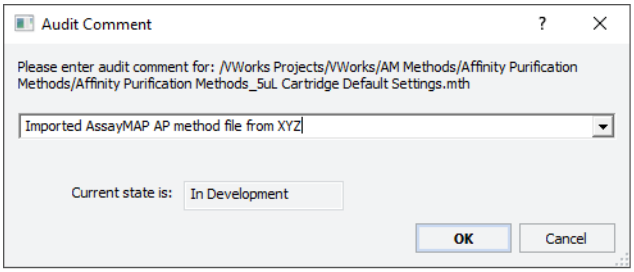
   You can remove the subfolder name from the method file name after you have selected the correct app or utility subfolder.

   c Ensure that the **Files of type** box specifies **AssayMAP Method Files (*.mth)**, and then click **Save**.

   If a file of the same name already exists in this folder, a message tells you that you cannot overwrite the existing file.

4 *VWorks Plus only*. The Audit Comment dialog box opens. Select or type the audit comment, and then click **OK**.

Note: Imported AssayMAP method files are exempt from changes in record state. An audit trail is always logged for exempt state records.



2 Using Experiment IDs

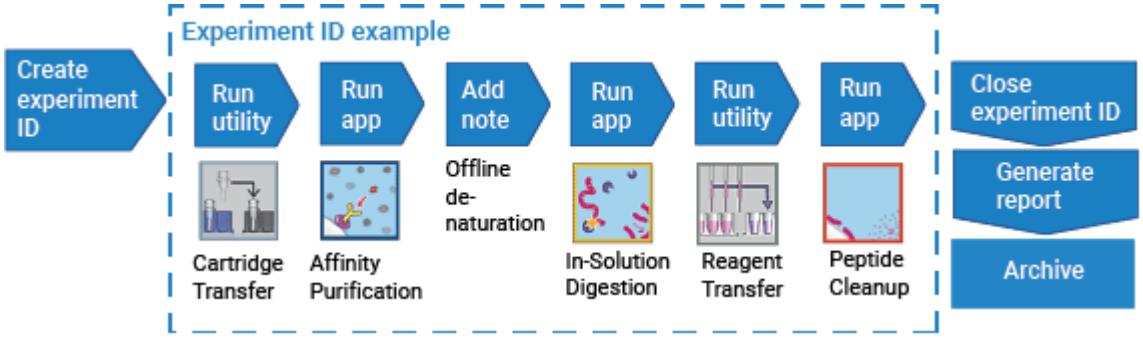
This section contains the following topics:

Experiment IDs and database overview

An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded. You use the Experiments Editor to create and manage experiment IDs.

The following figure shows an example of how an experiment ID can be used to record the steps performed and settings used during the course of a sample prep workflow.

Figure Example of an Experiment ID workflow



Experiment IDs are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

Software prerequisites

Before the Experiments Editor is available for use, a connection to the VWorks Experiments database must be established. For instructions, see "Setting up and connecting VWorks Experiments database" on page 101 in the *VWorks Automation Control Setup Guide*.

Workflow overview for experiment IDs

Step	For this task...	See...
1	Use Experiments Editor to create an experiment ID. *Note:* You can open the Experiments Editor from any Workbench app or utility.	"Creating and managing experiment IDs" on page 26
2	In the app or utility, select the experiment ID and method, and then start the run. During the protocol run, the software updates the database with the specified information for the selected experiment ID.	User guide for the workbench app or utility

Step	For this task...	See...
3	If applicable, add notes while the experiment ID has an Open status. The preceding figure shows an example of a note for a denaturation step.	"Adding notes to an experiment ID" on page 30
4	Generate an experiment ID report.	"Generating an experiment ID report" on page 32
5	Close the experiment ID. An experiment ID report is automatically generated.	"Closing an experiment ID" on page 35
6	Archive closed experiments to help prevent the Experiments database from being overloaded.	"Closing and archiving an experiment ID" on page 35
7	Periodically make a backup copy of the Experiments database and store the backup in a secure location. Ensure that the backup copy includes: • Exported data currently in the Experiments database • Archived experiment ID data.	"Exporting and importing experiment IDs" on page 42

Creating and managing experiment IDs

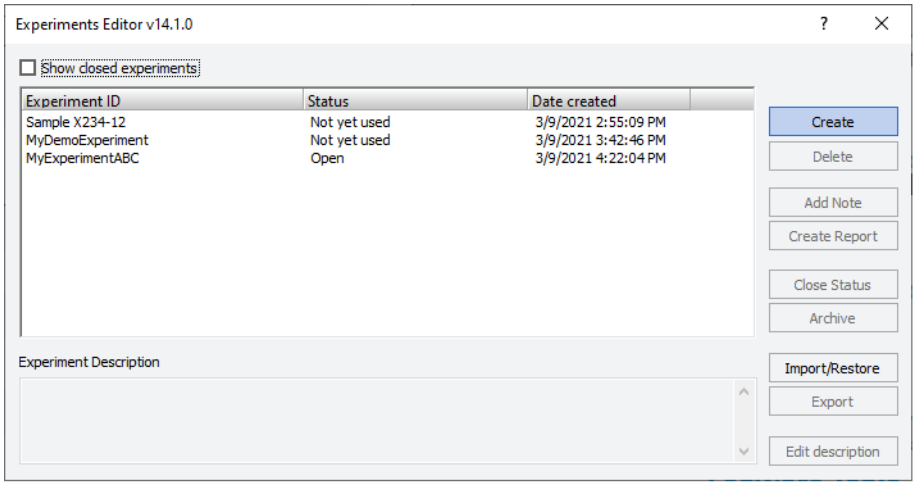
You must have VWorks administrator or technician privileges to create experiments.

Creating an experiment ID

To create an experiment ID:

1 In the navigation pane of an app or utility, click **Experiments Editor**. The Experiments Editor window opens.

2 In the **Experiments Editor** window, click **Create**.



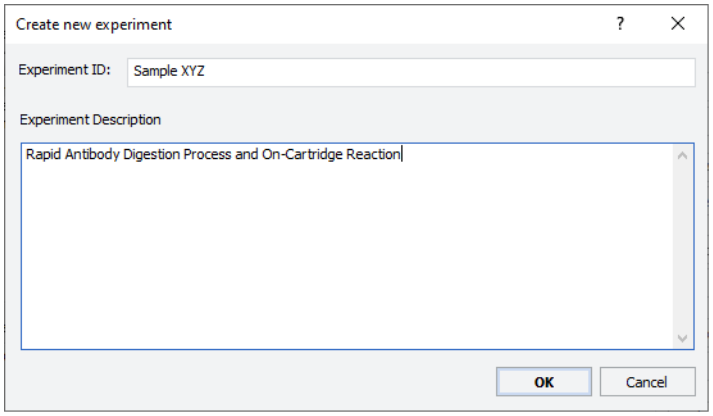
3 In the **Create New Experiment** dialog box that opens:

a Type a name in the **Experiment ID** box.

b In the **Experiment Description** box, type a description for this experiment ID.

c Click **OK**.

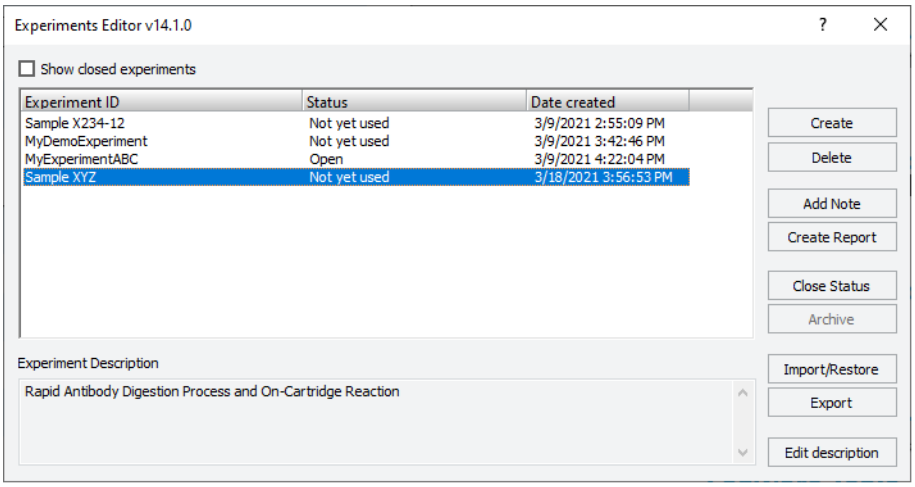
Note: The software assigns each experiment ID a hidden unique identifier (GUID).



4 In the **Experiments Editor** window, notice the following:

- The new experiment ID appears in the Experiment ID table.

- The Status column displays
  - **Not yet used** for any experiment IDs that have not been associated with any protocol runs.
  - **Open** if the experiment ID has had data added to it from at least one application run.
  - **Closed** for any closed experiment IDs only if the Show closed experiments check box is selected.
- The Experiment Description box displays the comments for the selected experiment ID.



Editing descriptions for an experiment ID

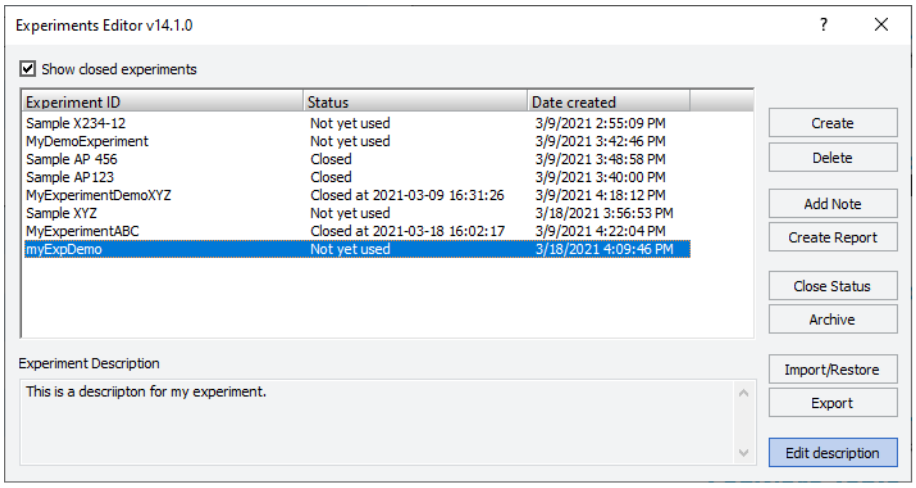
You may edit the descriptions of experiment IDs that have the Not Yet Used or Open status. Closed experiment IDs cannot be edited.

VWorks technician or administrator privileges are required for this procedure.

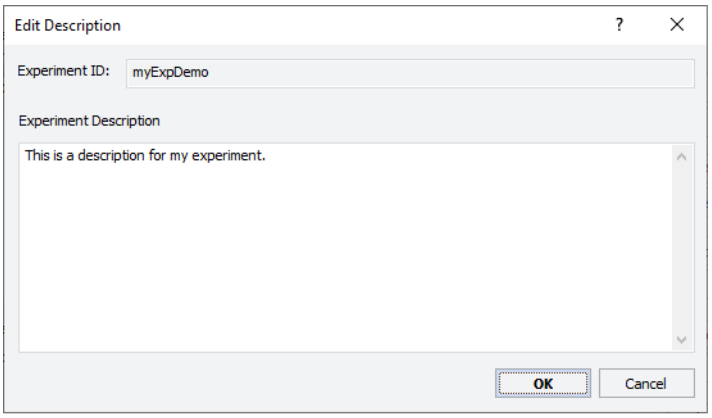
VWorks Plus only. You may edit the description of an experiment ID only if it has the Not Yet Used status.

To edit the description for an experiment ID:

1 In the **Experiments Editor** window, select the **Experiment ID**, and then click **Edit description**.



2 In the **Edit Description** dialog box, type or modify the description, and then click **OK**.



In the Experiments Editor window, select the experiment ID to view the corresponding description in the Experiment Description box.

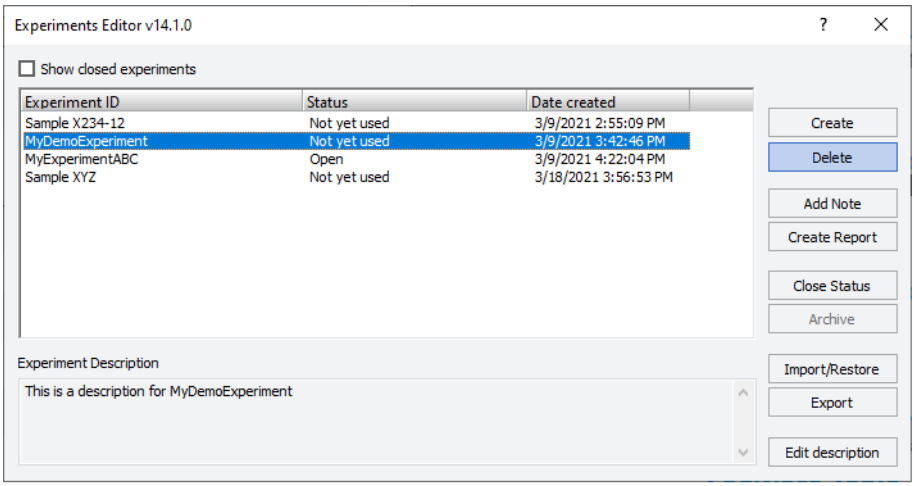
Deleting an experiment ID

An administrator may delete an experiment ID if it has a Not Yet Used or Open status. Closed experiments cannot be deleted.

VWorks Plus. Only an experiment ID with a Not Yet Used status can be deleted.

To delete an experiment ID:

1 In the **Experiment Editor** window, select the **Experiment ID**, and then click **Delete**.



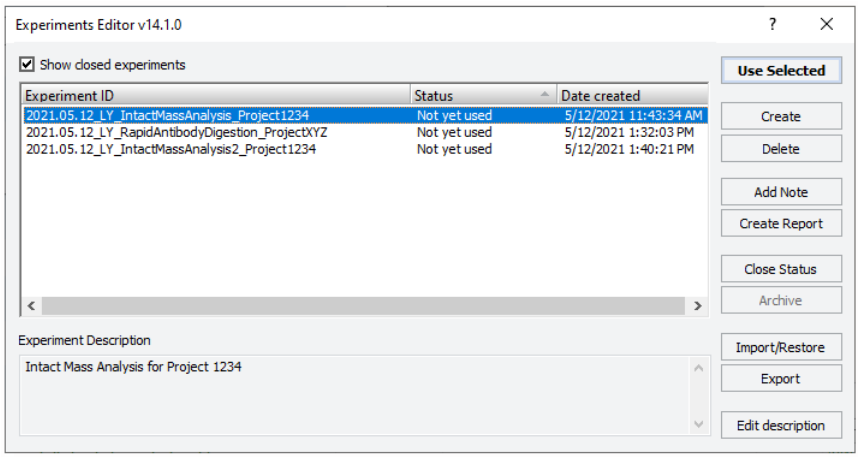
2 In the confirmation message that appears, click **Yes** to delete this experiment ID. The experiment ID no longer appears in the Experiment ID table.

Selecting an experiment ID and adding notes

Selecting an experiment ID for a run

To select an experiment ID for a run:

1. In a form, click the **Select Experiment** button. The Experiments Editor window opens.
2. In the **Experiments Editor** window, select the **Experiment ID** and then click **Use Selected**.



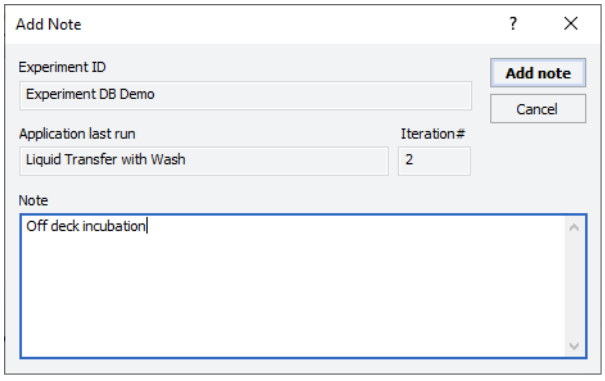
The selected experiment ID will be used for the next run initiated from the form.

Adding notes to an experiment ID

You can add notes to an open experiment ID, for example, to describe an off-deck denaturation step. The notes that you add will appear in any reports generated for the experiment ID.

To use the Add Experiment Note button:

1. Ensure that the **Experiment ID** is selected in form, and then click Add Experiment Note. The Add Note dialog box opens.

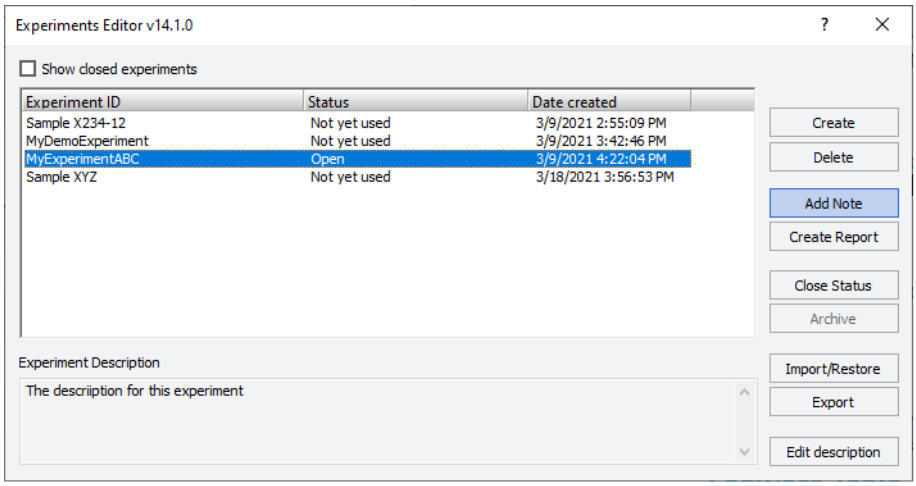


2 In the **Note** area, type the note, and then click **OK**.

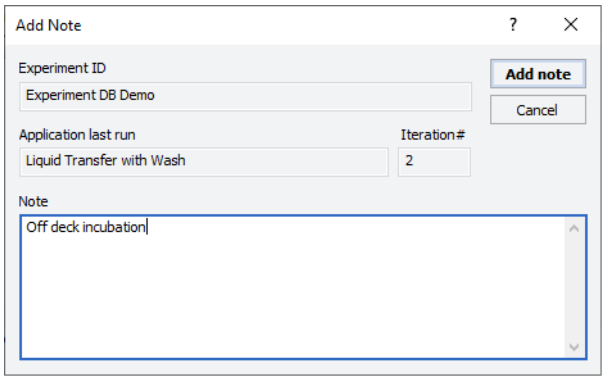
To select an experiment ID and add a note:

1 Do one of the following to open the Experiments Editor:
- In an app or utility form, click **Select Experiment**.
- In the VWorks window, click **Tools > Experiments Editor**.

2 In the **Experiments Editor** window, select the **Experiment ID**, and then click **Add Note**.



The Add Note dialog box opens.



3 In the **Note** area, type the note, and then click **OK**.

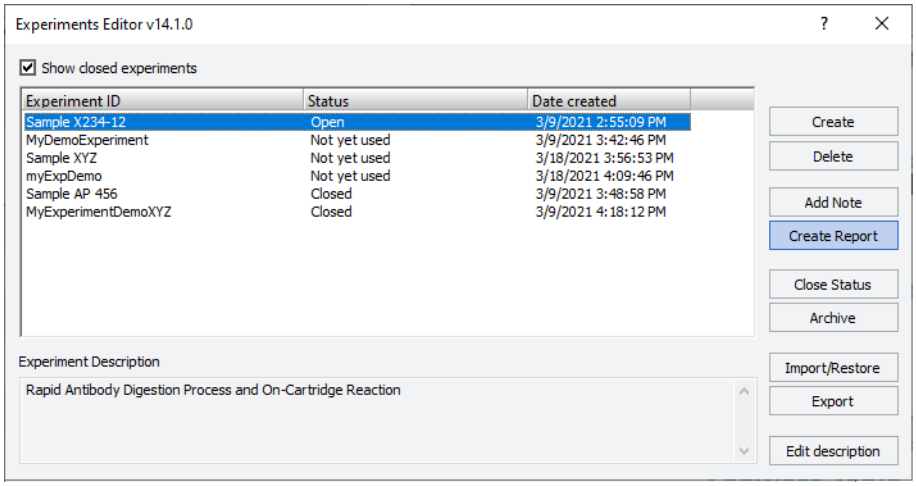
Generating an experiment ID report

The software automatically generates an experiment ID report when the experiment ID is closed. The following procedure describes how to generate a report for any experiment ID selected in the Experiments Editor.

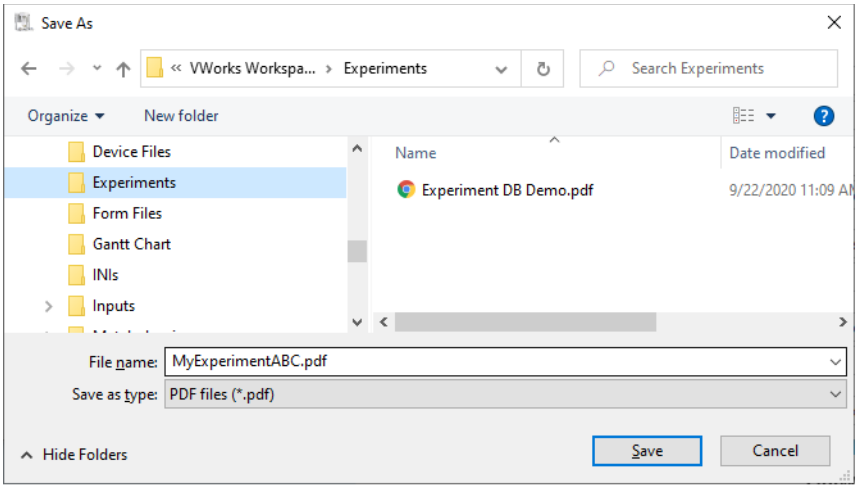
Creating a report for a selected experiment ID

To create an experiment ID report:

1. Open the **Experiments Editor** using one of the following methods:
   - In a VWorks form, click the **Experiments Editor** or **Select Experiment** button.
   - In the VWorks window, click **Tools > Experiments Editor**.
2. *Optional*. To include closed experiment IDs in the Experiments Editor window, select the **Show closed experiments** check box.
3. Select the **Experiment ID** and then click **Create Report**.



4. In the **Save As** dialog box, type a file name for the report, select a storage location, and then click **Save**. The software saves the report as a pdf file.



Note: The VWorks software uses the Microsoft Print to PDF virtual printer to generate .pdf files. This virtual printer is installed on all Windows 10 systems by default. If the virtual print has been removed, the report generation feature will not work.

Experiment ID report contents

The following figure shows an example of an experiment ID report, the following table describes the report contents.

Figure Example experiment ID report

```
Report generated at FEB-02-2022 06:44:55
Experiment "2022.02.02 Affinity Purification", created at FEB-02-2022 06:41:36 by user
admin
Description:



Application "Affinity Purification v3.0", iteration 1 (simulated), launched at
FEB-02-2022 06:43:21 by user admin
---------------------------------------------------------------------

Step "Initial Setup", started at FEB-02-2022 06:43:25, completed at FEB-02-2022
06:43:25 :
Method: 25uL Cartridge Default Settings.mth
Cartridge Type: 25µL Cartridges
Number of Full Columns of Cartridges: 1
1. Wash Station:  96AM Tip Wash Station
2. Seating Station + Cartridges:  96AM Cartridge Seating Station
3. Priming and Equilibration Buffer Labware: 96 AbGene 1127, 1mL Deep Well, Square
Well, Round Bottom
4. Sample Plate Labware: 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5. Cartridge Wash Buffer 1 Labware: 12 Column, Low Profile Reservoir, Natural PP
6. Cartridge Wash Buffer 2 Labware: 12 Column, Low Profile Reservoir, Natural PP
7. Flow Through Collection Labware: 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8. Elution _Syringe Wash Buffer Labware: 12 Column, Low Profile Reservoir, Natural PP
9. Eluate Collection Labware: 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Step "Initial Syringe Wash", started at FEB-02-2022 06:43:33, completed at FEB-02-2022
06:43:33 :
Conduct Step: yes
Wash Cycles: 3

Step "Prime", started at FEB-02-2022 06:43:34, completed at FEB-02-2022 06:43:34 :
Conduct Step: yes
Volume: 250
Flow Rate: 300
Wash Cycles: 1

Step "Equilibrate", started at FEB-02-2022 06:43:37, completed at FEB-02-2022 06:43:37
:
Conduct Step: yes
Volume: 250
Flow Rate: 10
Wash Cycles: 1
```

Table Report contents

Item	Description
Report header	Date that the report was generated • Experiment ID, creation date, and user log in of experiment ID creator • Description provided for the experiment
Archival status and history, if applicable	Archival status of experiment ID: • Date of archival or restoration • User who archived or restored • File name and path of the archived to or restored from location
Application and utility details	• Application or utility name • Iteration of the protocol run, and if it was simulated. Each iteration is listed separately, and ordered by launch time. Individual steps within an application or utility iteration are separated by a single blank line. • Log in of the user • Date and time run was started • Method name • Number of full columns of cartridges • Labware selection for each deck location • Step details for each step in the application
Notes	• Date and time the note was appended and the user login • Note contents

Closing and archiving an experiment ID

You must have VWorks administrator or technician privileges to change the status of an experiment ID from open to closed or from closed to archived.

VWorks Plus. Only a VWorks administrator may archive an experiment ID.

Experiment ID status	Description
Closed	• Automatically generates an experiment ID report and stores it in Shared Services storage. The experiment ID is used as the file name of the report. • Cannot be reopened. • Cannot have data added and it cannot be used for any subsequent application runs. If e-signatures are enabled, closed status is pending until all required signatures are completed. While closed status is pending, the experiment ID cannot have data added and it cannot be used for any application runs.
Archived	• Saves experiment ID or IDs to a file and erases associated experiment ID data from the active Experiments database. You can archive individual or multiple experiment IDs to a given archive file. • May be restored to the Experiments database as a closed experiment ID or IDs. • Helps prevent the Experiments database from being overloaded. – You should periodically archive closed experiment IDs that are no longer active, and keep a backup copy in a secure location. – You may only archive closed experiment IDs. – You cannot edit, delete, or export archived experiments.

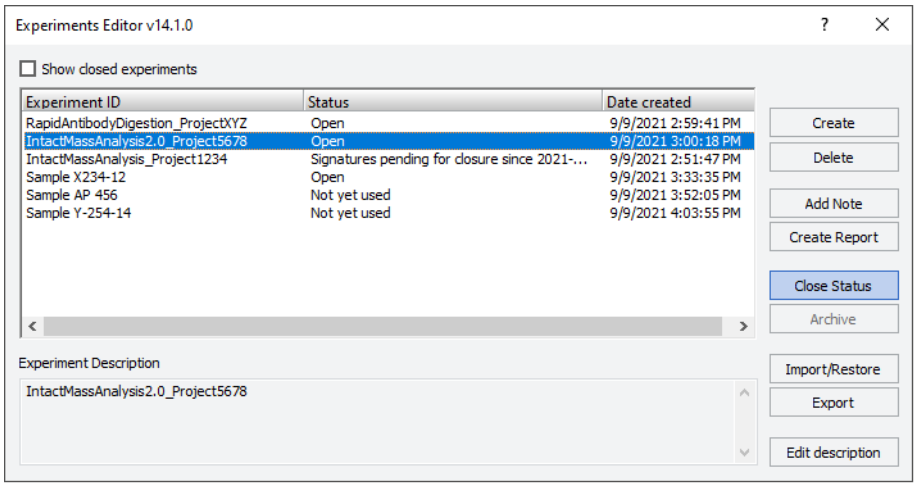
IMPORTANT

To ensure that you can recover the experiment ID data if the computer crashes or data get corrupted or lost, keep a backup copy of all data from the Experiments database and any archived experiment ID files in a secure location.

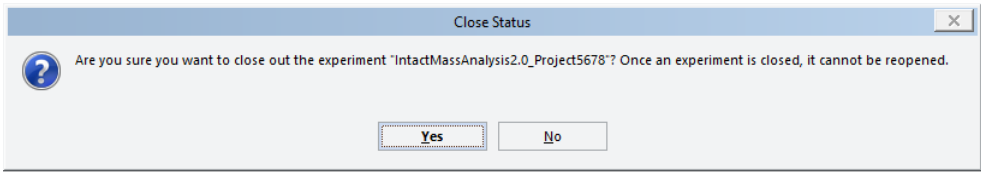
Closing an experiment ID

To close an experiment ID:

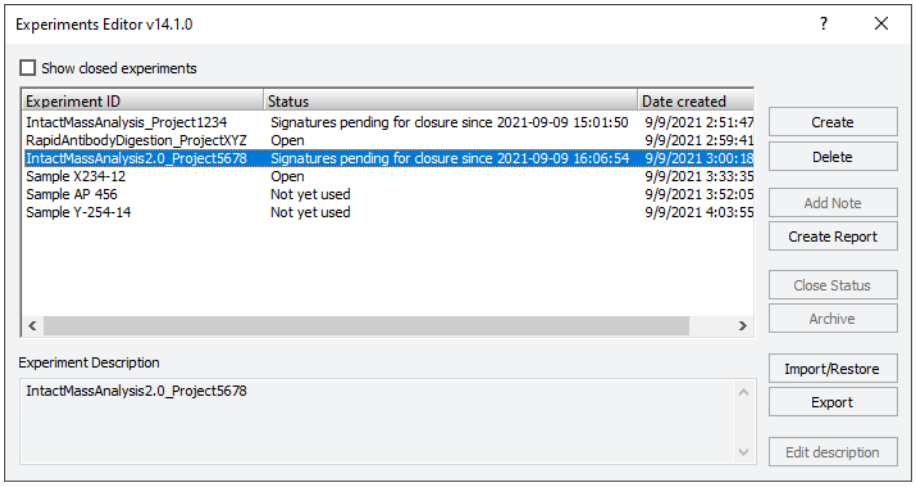
1 In the **Experiment Editor**, select the **Experiment ID** that you want to close. It must have an Open status. Click **Close Status**.



A message warns you that a closed experiment ID cannot be reopened. Click **Yes** to continue.

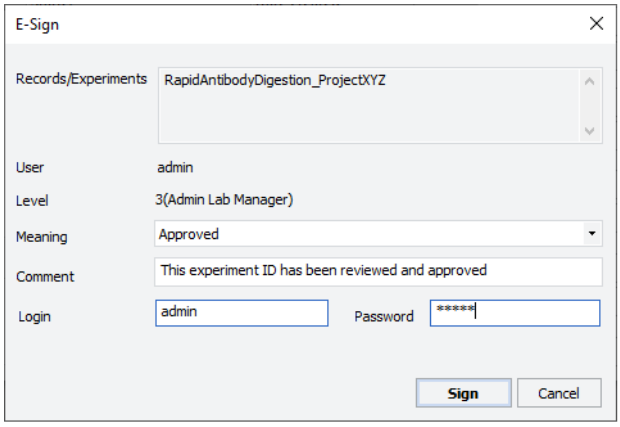


VWorks Plus. If e-signatures are enabled, the status changes to Signatures pending for closure since *<date time>*. Only after all the required signatures are completed will the status change to Closed.



2 *VWorks Plus only*. If e-signatures are enabled and your login credentials allow you to sign at this stage, enter the following in the **E-Sign** dialog box, and then click **Sign**:

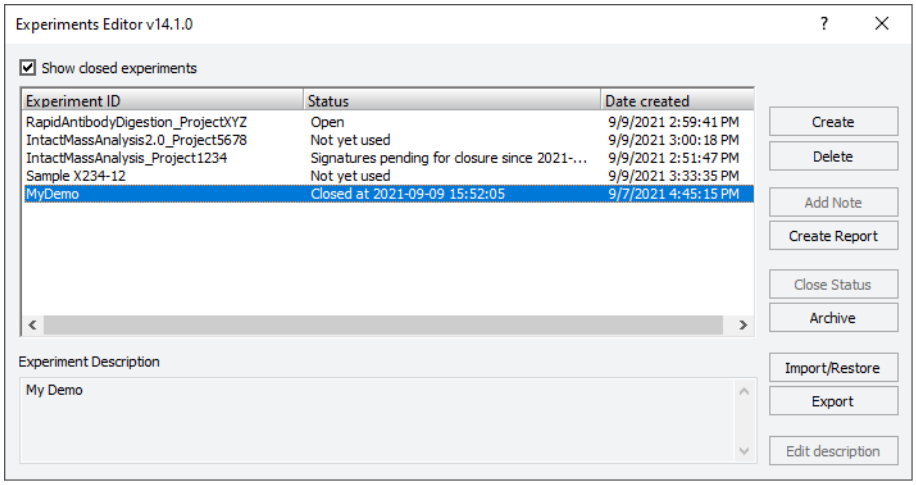
- **Meaning.** Select the meaning from the list.
- **Comment**. Type a comment about why you are closing this experiment ID.
- **Login** and **Password**. Type your VWorks login credentials.



A Signature(s) posted successfully message displays.

If you do not have the credentials to sign at this stage, you may want to notify the other signatories that their signatures are pending.

3 To view closed experiment IDs in the **Experiments Editor**, select the **Show closed experiments** check box at the top of the window.



The Status column displays Closed at <*date and time*> for the closed experiment IDs. If the experiment ID was restored from an archive, the Status column displays Closed without the date-time stamp.

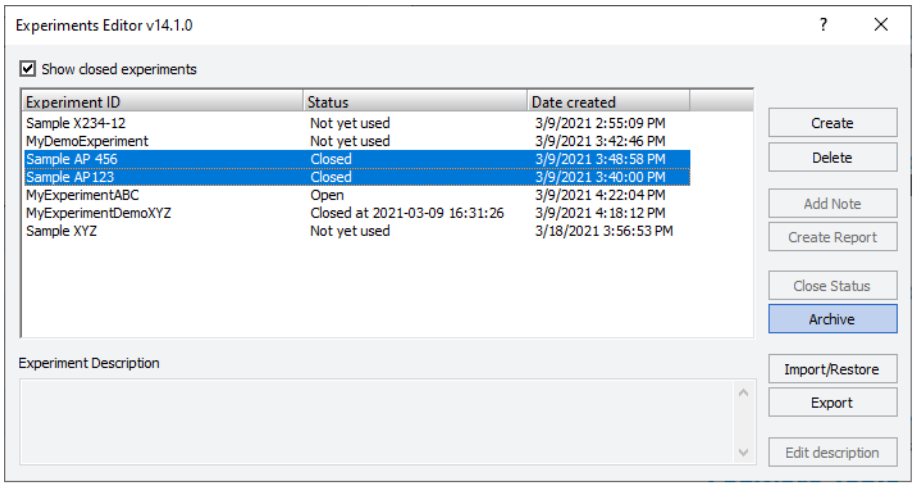
Archiving an experiment ID

To archive an experiment ID:

1 In the **Experiments Editor** window, select the **Show closed experiments** check box.

2 Select the **Closed** experiment ID or IDs to be archived, and then click **Archive**.

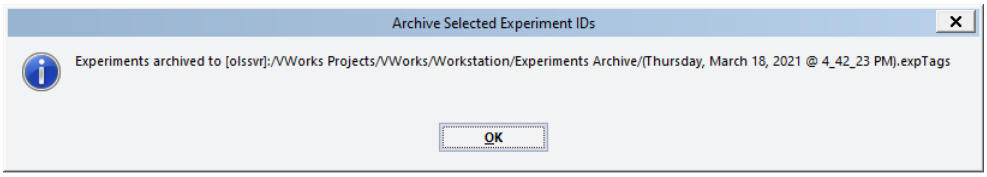
Note: To select multiple items, use SHIFT+click or CTRL+click.



3 When the confirmation message opens stating that all data associated with the archived experiment ID or IDs will be saved to a file and that the data will be erased from the database, do one of the following:

- Click **No** to cancel the archive operation.
- Click **Yes** to archive the selected experiment IDs.

  An Archived Selected Experiment IDs message opens and lists the storage location and file name (<*datetimestamp*>.expTags), as the following example shows.

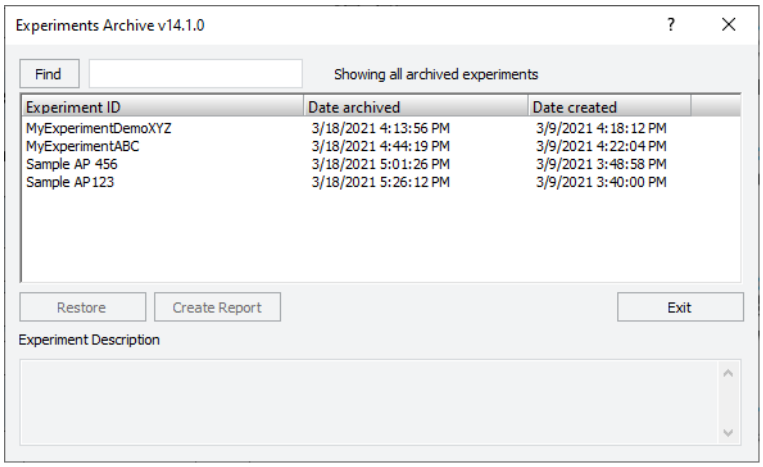


Viewing the archived experiment IDs

To view the archived experiment IDs:

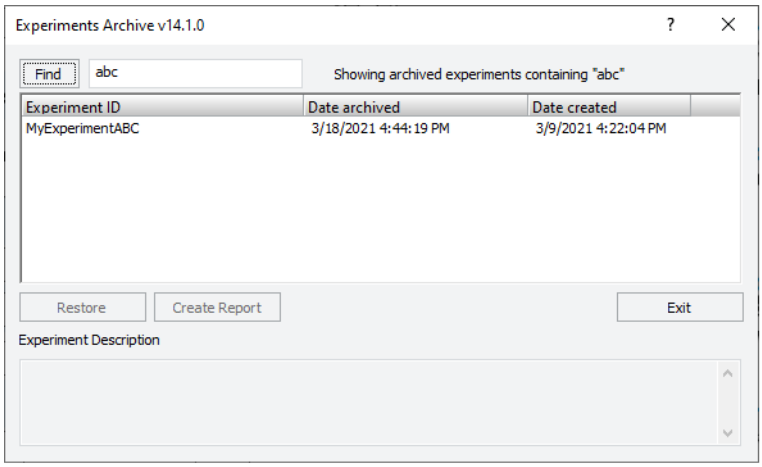
1 In the VWorks window, click **Tools > Experiments Archive**.

The Experiments Archive dialog box opens and displays all archived experiment IDs.



2 To filter the list of archived experiment IDs, type the filter text in the **Find** box, and then click **Find**.

This filter is not case-sensitive as the following example shows.



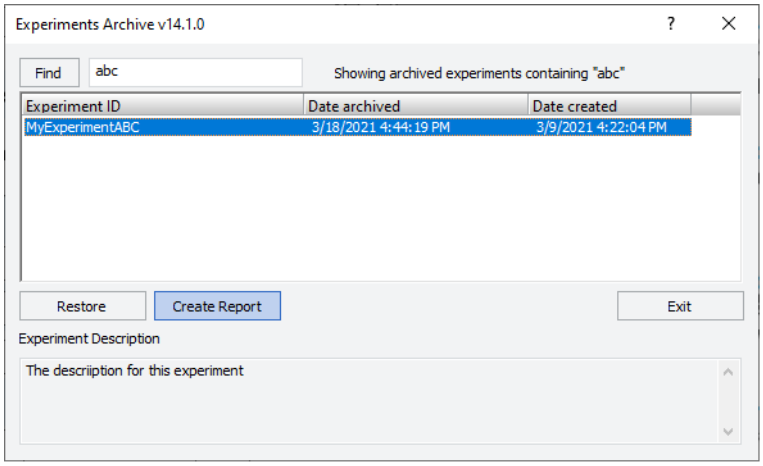
3 Select the experiment ID to view the description in the Experiment Description box.

Creating reports of archived experiment IDs

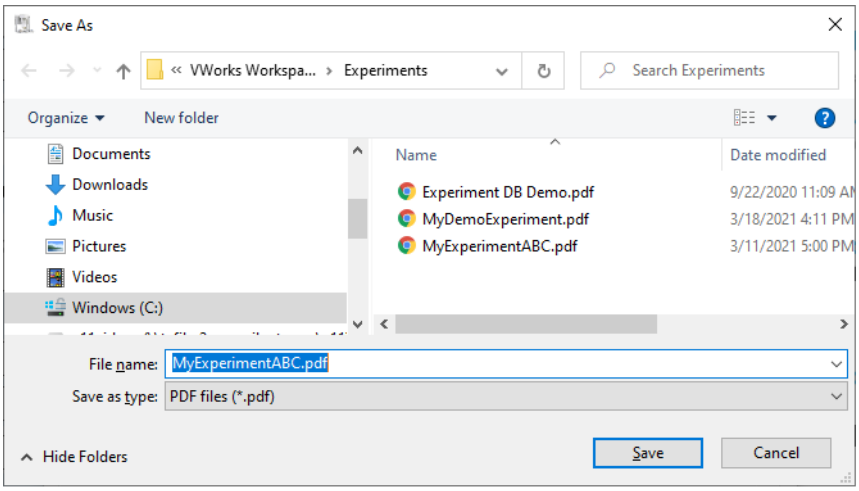
To create a report for an archived experiment ID:

1 In the VWorks window, click **Tools > Experiments Archive**.

2 In the **Experiments Archive** dialog box, locate and select the **Experiment ID**, and then click **Create Report**.



3 In the **Save As** dialog box, specify the file name and storage location, and then click **Save**.



Restoring archived experiment IDs to the database

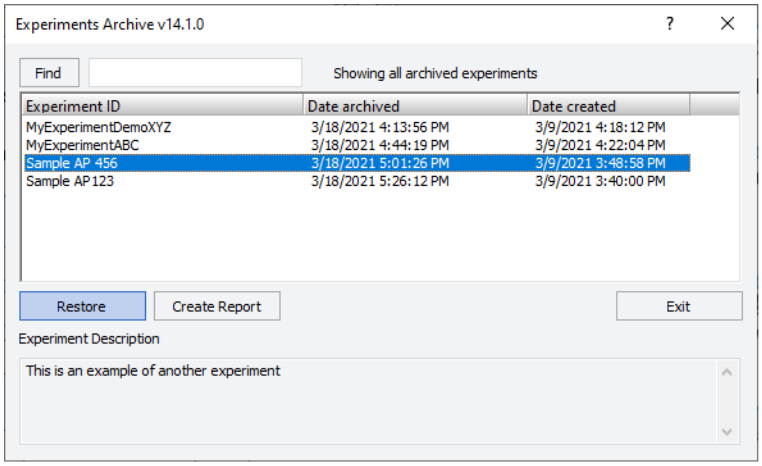
You must have VWorks technician or administrator to restore archived experiment IDs.

When you restore an archived experiment ID to the database, the status of the experiment ID changes to closed.

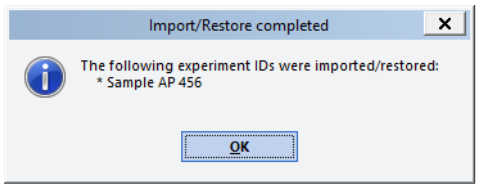
To restore archived experiment IDs to the Experiments database:

1 In the VWorks window, click **Tools > Experiments Archive**.

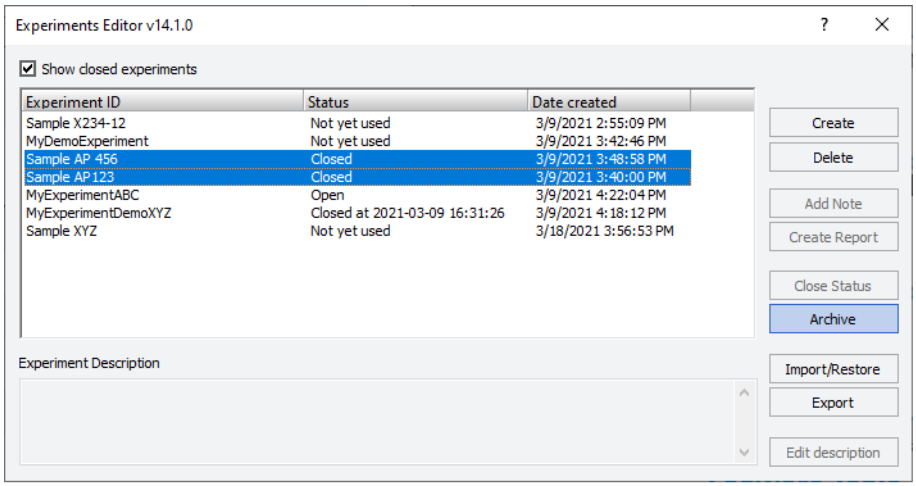
2 In the **Experiments Archive** dialog box, locate and select the experiment ID or IDs, and then click **Restore**.



The Import/Restore Completed message appears and lists the restored experiment IDs.



The Experiments Editor lists the restored experiment IDs with a Closed status and no date-time stamp.



Exporting and importing experiment IDs

IMPORTANT To ensure that you can recover the experiment ID data if the computer crashes or data get corrupted or lost, keep a backup copy of all data from the Experiments database and any archived experiment IDs in a secure location.

You can export active experiment IDs from the Experiments database to files in the local file system. You can export the data for individual or multiple experiment IDs to a single file. The exported data does not include archived experiment IDs.

When importing any previously exported experiment IDs, the software performs tamper detection to verify that no changes were made to the exported experiment ID files.

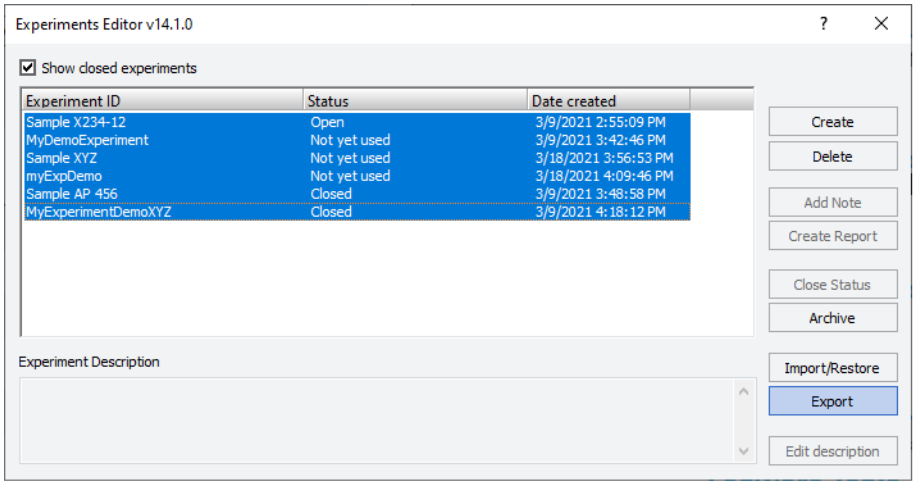
You should periodically export the data for all active experiment IDs to create a backup copy of the database. You can import the backup copy to recover the experiment ID data in case the data become damaged or lost.

Exporting experiment IDs

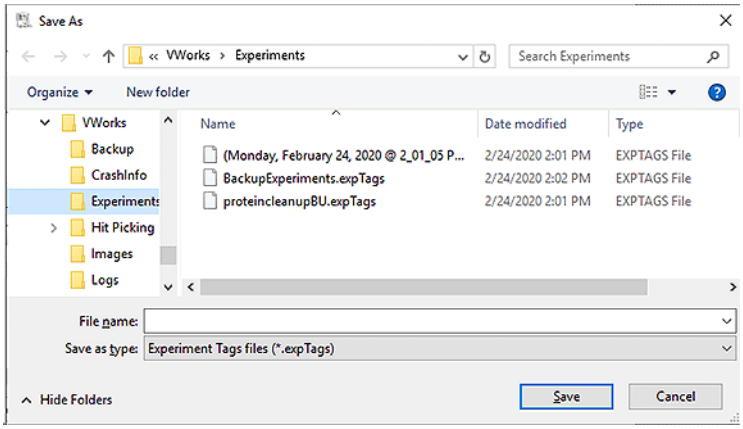
You can export experiment IDs that have a status of Not Yet Used, Open, or Closed.

To export experiment IDs:

1. Do one of the following to open the Experiments Editor:
   - In an app or utility form, click **Experiments Editor** or **Select Experiment**.
   - In the VWorks window, click **Tools > Experiments Editor**.
2. In the **Experiments Editor** window, select the experiment IDs, and then click **Export**.



3. In the **Save As** dialog box, select the storage location, type a file name, and then click **Save**.



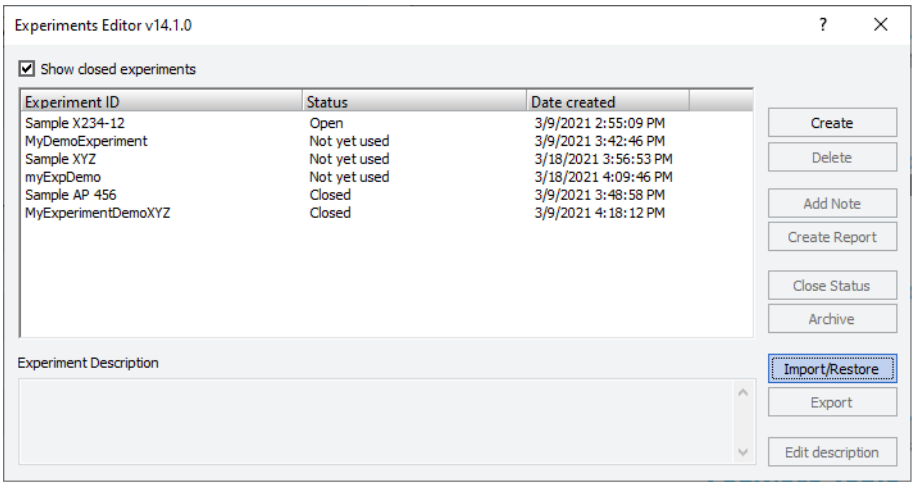
The software exports all the data for the selected experiment IDs to an *.expTags file, which is in XML format.

Importing experiment IDs

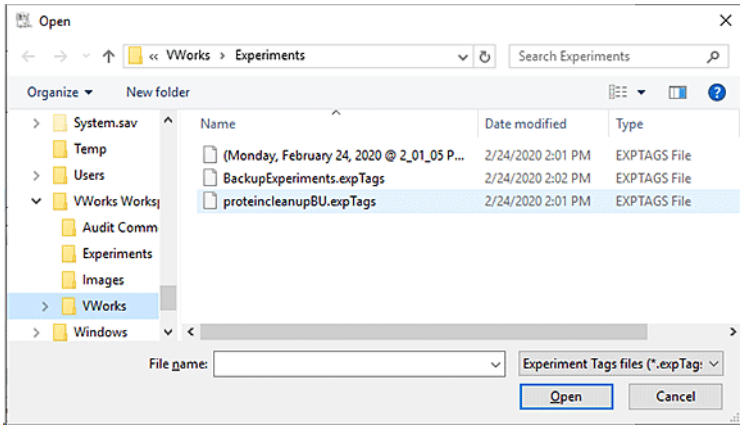
You can import the experiment IDs from a previously saved *.expTags file, for example as part of a data recovery process. However, you cannot import an experiment ID if it already exists in the Experiments database.

To import experiment IDs:

1. Do one of the following to open the Experiments Editor:
   - In an app or utility form, click **Experiments Editor** or **Select Experiment**.
   - In the VWorks window, click **Tools > Experiments Editor**.
2. In the **Experiments Editor** window, click **Import/Restore**.

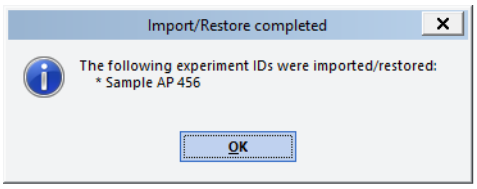


The Open dialog box appears.



3 Select the file (*.expTags) that contains the experiment IDs to be imported, and then click **Open**.

The Import/Restore Completed message appears and lists the import experiment IDs. The message also lists any experiment IDs that were not imported because they already exist in the Experiments database.



3 Affinity Purification v3.0 User Guide

This chapter contains the following topics:

Note: This section presents instructions for using the Affinity Purification v3.0 application. If you are using the Aspiration Mode version, see "Affinity Purification: Aspiration Mode v3.0 User Guide" on page 89.

App description

Affinity Purification v3.0. This application enables automated affinity purification of target molecules, such as antibodies and peptides, from 1 to 96 samples in a single run.

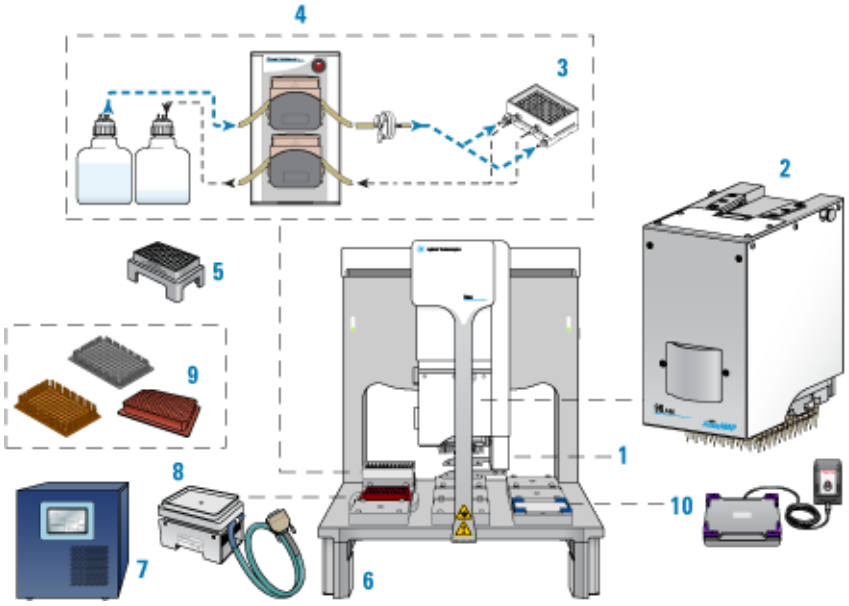
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Affinity Purification protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Affinity Purification on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Protein A (PA-W) cartridge rack	G5496-60000	G5496-60018
AssayMAP Protein G (PG-W) cartridge rack	G5496-60008	–
AssayMAP Streptavidin (SA-W) cartridge rack	G5496-60010	G5496-60021
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of Protein A, Protein G, or Streptavidin 5- or 25-µL cartridges are required. For details, see Preparing the sample plates.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.
- PA-W, SA-W, and PG-W cartridges tolerate brief exposure to pH as low as 2.0. The stability of the PA-W, SA-W, and PG-W cartridges after capturing additional affinity ligands should be determined empirically.

Labware

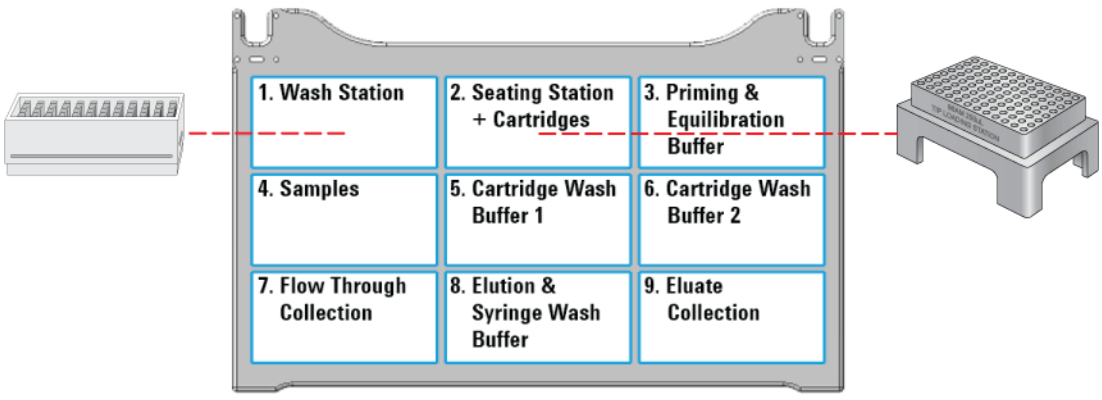
Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3, 5, 6, 7, 8

Labware	Manufacturer part number*	Deck location options
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3, 5, 6, 7, 8
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—9
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7, 9**
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3—9
96 Greiner 675801, Half Area, Flat Bottom, UV Star	Greiner 675801	4, 7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	3, 5, 6, 8

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner PCR plate is not compatible with the 25 µL cartridges at deck locations 7 and 9.

Reagents

The volume, type, and concentration of reagents required for affinity purification vary depending on sample characteristics and the desired analytical result. Consult the resources that cover general principles of affinity purification such as those listed in the "Reference library" on page 87. For examples of reagents used with specific affinity interactions, consult the published scientific literature including publications that use the AssayMAP Bravo listed in the Agilent AssayMAP Bravo Citation Index.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure due to the buildup of salts within the syringe barrels. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see "Syringe Wash v3.0 User Guide" on page 567.

All labware require volume overage for the protocol to execute properly. Use the Affinity Purification Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 51.

Preparing the solutions

The following solutions are required for the Affinity Purification protocol:

- Priming & Equilibration Buffer
- Cartridge Wash Buffers
- Elution & Stringent Syringe Wash Buffer

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Affinity Purification

The Reagent Volume Calculator is a Microsoft Excel file that contains the following:

- *Calculator worksheet*. You enter the number of columns to process, whether to perform the Collect Flow Through option, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

  Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.
- *Reagent Recipe worksheet*. You enter the concentrations of each component in your reagent, and the worksheet calculates the recipe volumes required.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.

5 Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figures show the worksheets of the Reagent Volume Calculator.

Figure Affinity Purification Calculator worksheet

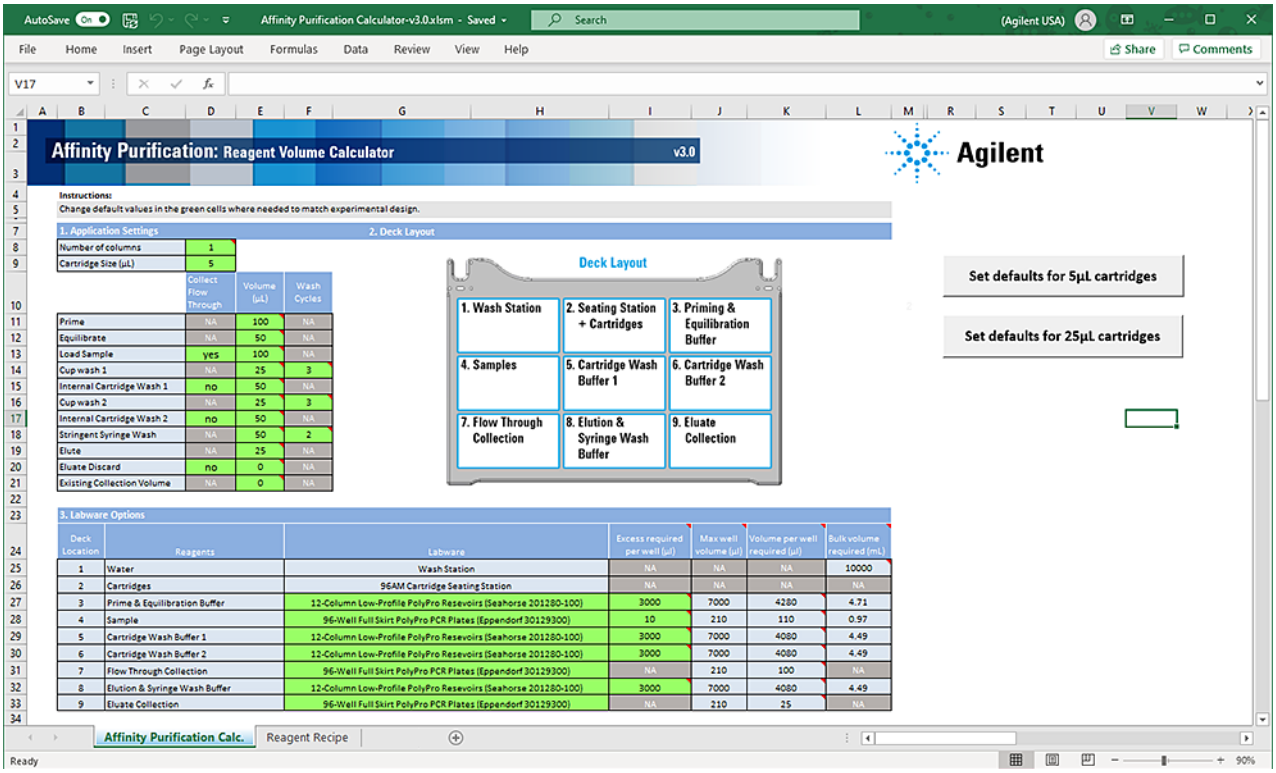
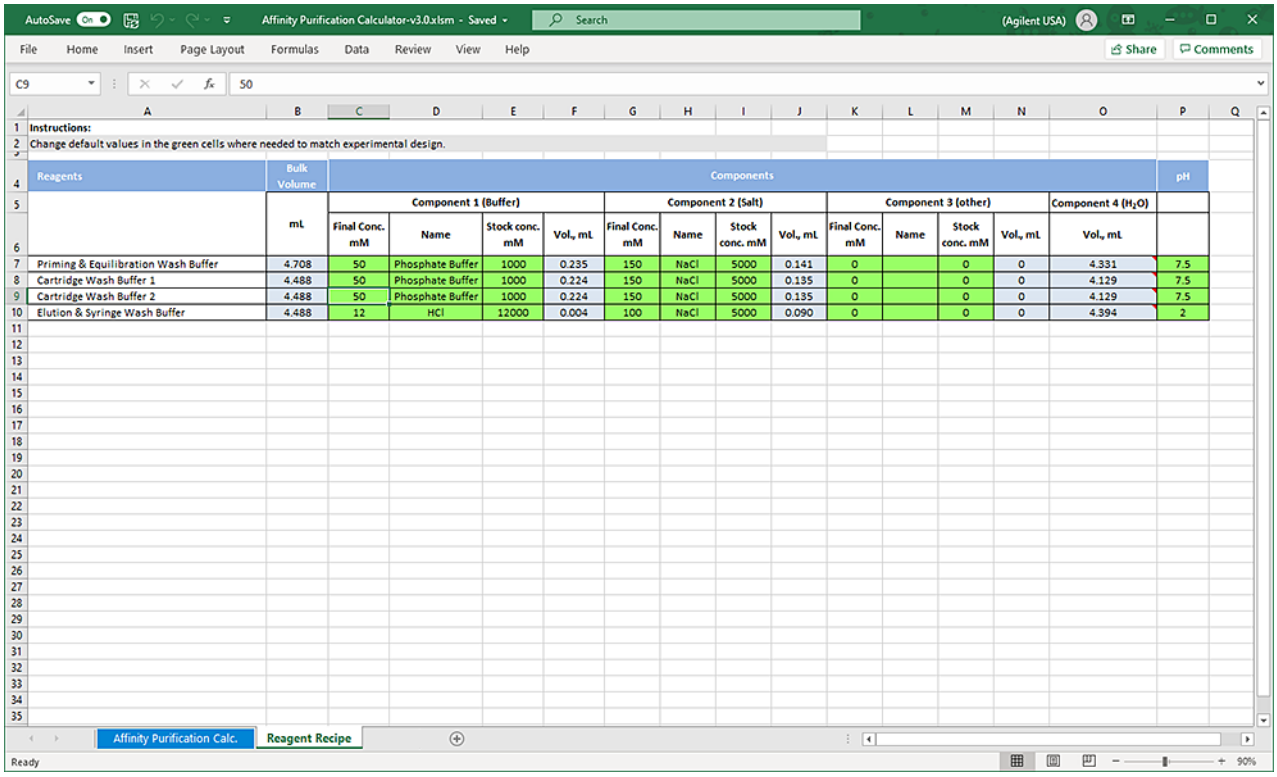


Figure Affinity Purification Reagent Recipe worksheet



Instructions:
Change default values in the green cells where needed to match experimental design.

Reagents	Bulk Volume	Components													pH
		Component 1 (Buffer)				Component 2 (Salt)				Component 3 (other)				Component 4 (H_2O)	
	mL	Final Conc. mM	Name	Stock conc. mM	Vol., mL	Final Conc. mM	Name	Stock conc. mM	Vol., mL	Final Conc. mM	Name	Stock conc. mM	Vol., mL	Vol., mL	
Priming & Equilibration Wash Buffer	4.708	50	Phosphate Buffer	1000	0.235	150	NaCl	5000	0.141	0		0	0	4.331	7.5
Cartridge Wash Buffer 1	4.488	50	Phosphate Buffer	1000	0.224	150	NaCl	5000	0.135	0		0	0	4.129	7.5
Cartridge Wash Buffer 2	4.488	50	Phosphate Buffer	1000	0.224	150	NaCl	5000	0.135	0		0	0	4.129	7.5
Elution & Syringe Wash Buffer	4.488	12	HCl	12000	0.004	100	NaCl	5000	0.090	0		0	0	4.394	2

Preparing the buffers

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the AssayMAP affinity purification chemistry.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming & Equilibration Buffer (deck location 3)	Typically a buffered aqueous solution with neutral pH and physiologic salt concentration, such as Phosphate-Buffered Saline (PBS), that is similar in composition to the buffer solution used to prepare the sample.

Reagent (deck location)	Composition and comments
Cartridge Wash Buffer 1 (deck location 5)	High-stringency buffer (for example, a neutral buffer with high NaCl) or a low-stringency buffer (for example, PBS or a neutral mass-spec-friendly buffer) The Affinity Purification application is designed to allow the use of two wash buffers for high and low stringency washes or a single wash buffer. The buffer selection depends on a number of factors. First, consider whether Cartridge Wash Buffer 2 will or will not be used. This decision is often dependent on whether or not a stringent wash is desired in the protocol. If so, one would typically use the stringent buffer for Cartridge Wash Buffer 1 and then a less stringent wash buffer for Cartridge Wash Buffer 2, which would remove the stringent component of the buffer before elution. If a stringent buffer is not used, you would typically use Cartridge Wash Buffer 1 only, and it would be a low-stringency wash buffer, such as PBS or a neutral mass-spec-friendly buffer. *Note:* If you only do a single internal cartridge wash, it should be wash buffer 1, as skipping wash 1 and using wash 2 will result in skipping the sample chase which might decrease the amount of target recovered.
Cartridge Wash Buffer 2 (deck location 6)	Typically, a low-stringency buffer (for example PBS or a neutral mass-spec-friendly buffer) The composition of this buffer is often dictated by sensitivity of downstream steps to components of Wash Buffer 2 as the void volume in the cartridges (~2 µL for 5 µL cartridges or ~10 µL for 25 µL cartridges) will contain the last wash solution used before the Eluate step and end up in the eluate unless the Discard Eluate option is selected and this volume is discarded.
Elution & Stringent Syringe Wash Buffer (deck location 8)	12 mM HCl with 100 mM NaCl pH 2.0 or 5% acetic acid The elution solution is typically a low pH solution. A key consideration is if the sample will be neutralized following elution. If so, a 12 mM HCl solution with 100 mM NaCl is a good choice as it is very easy to neutralize. However, this solution requires approximately 4—6 column volumes to elute antibodies off the cartridge. If neutralization is not a key consideration or minimizing elution volume is a critical driver, a 5% acetic acid solution is a good choice because targets can be eluted in as little as 2 column volumes.

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and the cartridge positions in the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 55.

To dispense the solutions into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Priming & Equilibration Buffer into the labware to be placed at deck location 3.
3. Add the specified volume of Cartridge Wash Buffer 1 into the labware to be placed at deck location 5.
4. If using two wash buffers, add the specified volume of Cartridge Wash Buffer 2 into the labware to be placed at deck location 6.
5. Add the specified volume of Elution & Stringent Wash Buffer into the labware to be placed at deck location 8.
6. If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT

To minimize evaporation, prepare the samples immediately before running the Affinity Purification protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to optimize the binding conditions (for example, pH).
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the sample composition

The optimal chemical environment for binding is generally similar for protein A, protein G, and streptavidin.

- Protein A and G resins bind selectively to antibodies.

Examine the scientific literature for differences in their affinity for antibody subtypes from different species.

- Streptavidin resin binds selectively to biotinylated molecules.
- Protein A, protein G, and streptavidin are relatively unaffected by most sample components, including those present in complex protein mixtures.

What are optimal pH conditions?

One of the most important considerations for optimizing binding conditions is the pH of the sample, which should be near neutral pH. Both low (2 to 3) and high (10 to 11) pH ranges can prevent binding to protein A or protein G resins. In general, the sample should be:

- *Protein A*. Greater than pH 6.
- *Protein G*. Greater than pH 4.

What sample components cause concerns?

Protein A, protein G, and streptavidin generally tolerate moderate levels of salt, non-ionic detergents, and mild denaturing reagents, such as urea, quite well. You should examine scientific literature for the known effects and tolerances of protein A, protein G, and streptavidin, keeping in mind that these tolerances may differ depending on the antibody species and subtype.

Does the antibody species and isotype in the sample match the cartridge binding specificity?

Protein A and protein G bind a wide variety of antibody subtypes and species (1). Carefully consider the species and subtype of antibody when choosing between using the AssayMAP Protein A or Protein G cartridge for purification.

The antibodies that bind to protein G largely overlap the set that binds to protein A. While protein A is the industry standard for purification and titer determination of human therapeutic antibodies, protein G is the standard for purification of antibodies used as bioanalytical tools, primarily because many antibody subtypes are generated in species that bind poorly to protein A, for example, mouse IgG_1 and rat IgG_1 (2).

Determining the volume of sample to load

The AssayMAP Affinity Purification protocol permits loading up to 1000 µL of sample onto AssayMAP cartridges. For sample volumes > 250 µL, the protocol will iteratively load samples onto cartridges to stay within the maximum syringe volume (250 µL) of the Bravo 96AM Head.

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of the target molecule that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through. This value is dependent on the sample load flow rate.
- *Total binding capacity*. The maximum mass of the target molecule that can bind to the cartridge. This can only be achieved by loading significantly more of the target molecule than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity.

See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed information about the binding capacity for Protein A, Protein G, and Streptavidin cartridges.

What is the concentration of the target in the sample?

If you know the approximate concentration of the target molecule in your sample and you are working within the quantitative binding range of the cartridge, you can determine the volume of sample to load as follows:

$$\mu L \text{ sample to load} = \frac{\mu g \text{ target desired}}{\mu g/\mu L \text{ target in sample}}$$

Does the experiment require quantitative binding of the target?

For quantitative recovery of the target, the volume loaded must contain a mass of target protein that is equal to or less than the quantitative binding capacity. A bioprocess feed stream containing 10 mg/mL (10 µg /µL) antibody would require a maximum load volume of 10 µL (100 µg) to be within the quantitative range for 5 µL PA-W cartridges. If the antibody concentration is unknown, you can do multiple runs at different volumes (10, 100, and 1000 µL) to find a sample volume that is within the quantitative binding capacity, or a single run with the undiluted sample and multiple dilutions (no dilution, 1:10, and 1:100).

Preparing the sample plates

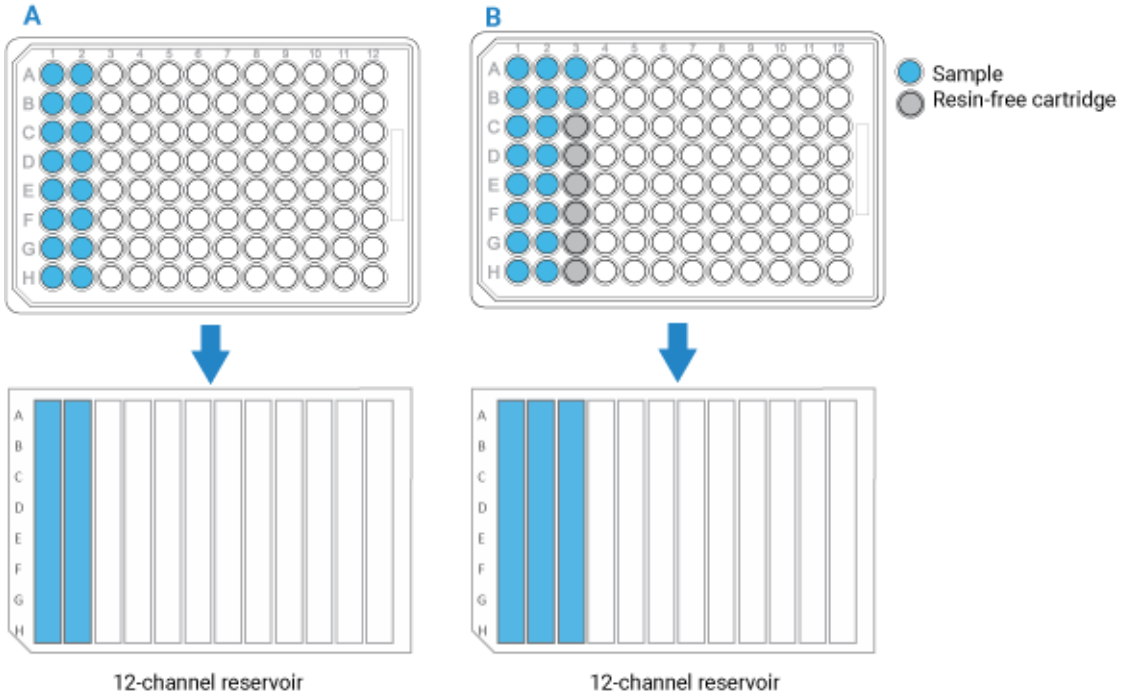
Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.

  The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based on the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use AssayMAP Resin-Free cartridges to fill the empty well positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash steps.

Figure Examples of sample microplate and reservoir layout: A) Multiple of 8 samples, B) Not a multiple of 8



See "Labware" on page 49 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION

A small volume excess is required in all labware types to ensure proper volume transfer.

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Affinity Purification protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the analyte from the cartridges.

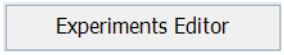
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the Cartridge & Tip Seating Station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 51.
- Prepare the samples. See "Preparing the samples" on page 55.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See "System Startup/Shutdown v3.0 User Guide" on page 574.
- Transfer the cartridges to the Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

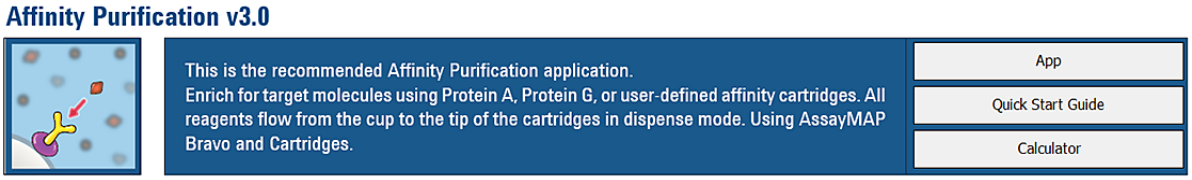
IMPORTANT Do not allow wetted cartridges to dry out. Agilent does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 48.

Setting up the protocol

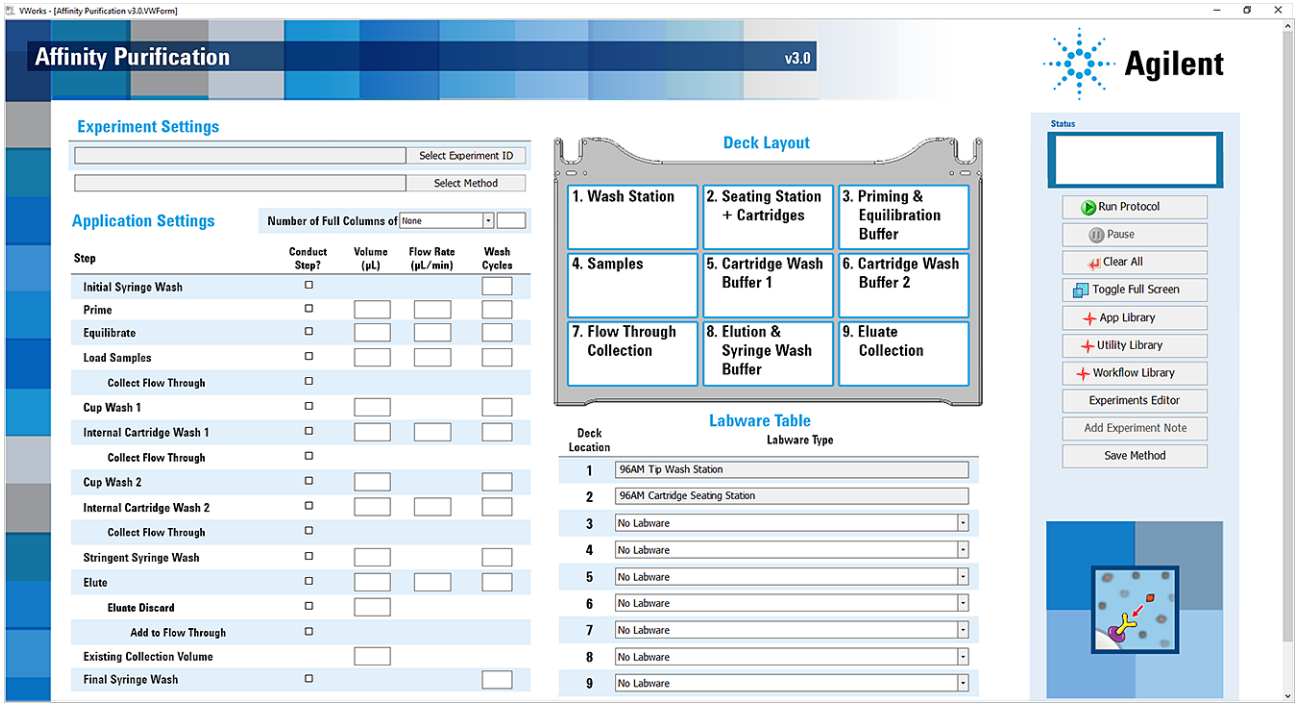
Before starting the protocol, make sure the appropriate selections and values are specified in the Affinity Purification application.

To set up the protocol:

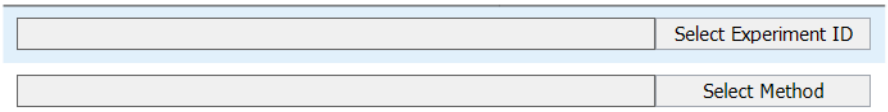
1. Open the **App Library**.
2. Locate **Affinity Purification**, and then click **App**.



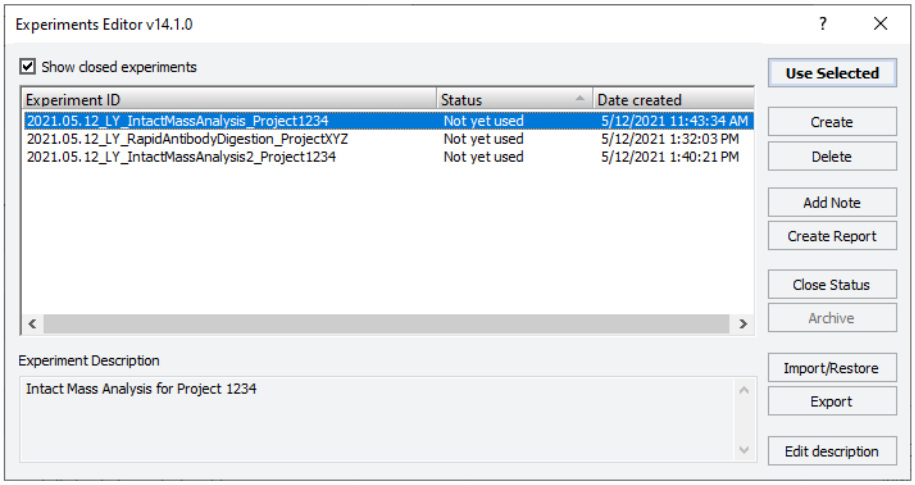
The Affinity Purification application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 65.
- To create or modify a method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:

a Select the cartridge size from the list:
- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION **If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.**

CAUTION **If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.**

IMPORTANT Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 68.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10
Equilibrate	Aspirates Equilibration Buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates samples (deck location 4) into the syringes, and then dispenses them through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–1000	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the sample flow-through at the wash station (deck location 1).	–	–	–	–
Cup Wash 1	Rinses the cartridge cups with Cartridge Wash Buffer 1 (deck location 5), and then discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Internal Cartridge Wash 1	Aspirates Cartridge Wash Buffer 1 (deck location 5) into the syringes, and then dispenses it through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash 1 flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the Internal Cartridge Wash flow-through at the wash station (deck location 1).	–	–	–	–
Cup Wash 2	Rinses the cartridge cups with Cartridge Wash Buffer 2 (deck location 6) and discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 2	Aspirates Cartridge Wash Buffer 2 (deck location 6) into the syringes, and then dispenses it through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash 2 flow-through at the Flow Through Collection plate (deck location 7). If not selected, discards the Internal Cartridge Wash 2 flow-through at the wash station (deck location 1).	–	–	–	–
Stringent Syringe Wash	Aspirates Syringe Wash Buffer (deck location 8) into the syringes, and then discards the liquid into the wash station (deck location 1).	5 µL:	50	–	2
		25 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates Elution Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Eluate Collection (deck location 9).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0–250	0.1–500	0–10
Eluate Discard	If selected, a specified initial volume of Eluate will be dispensed through the cartridges, and collected in the Flow Through Collection plate (deck location 7), or discarded at the wash station (deck location 1).	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–250	–	–
Add to Flow Through	If selected, collects the Eluate Discard in the Flow Through Collection plate (deck location 7). If not selected, discards the Eluate Discard at the wash station (deck location 1).	–	–	–	–

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Existing Collection Volume	Specifies the volume of liquid present in the Eluate Collection plate (deck location 9) at the beginning of the run.	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–1000	–	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 68.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 82.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT

The protocol will display an error message if cartridges are missing.

Starting the protocol run

WARNING

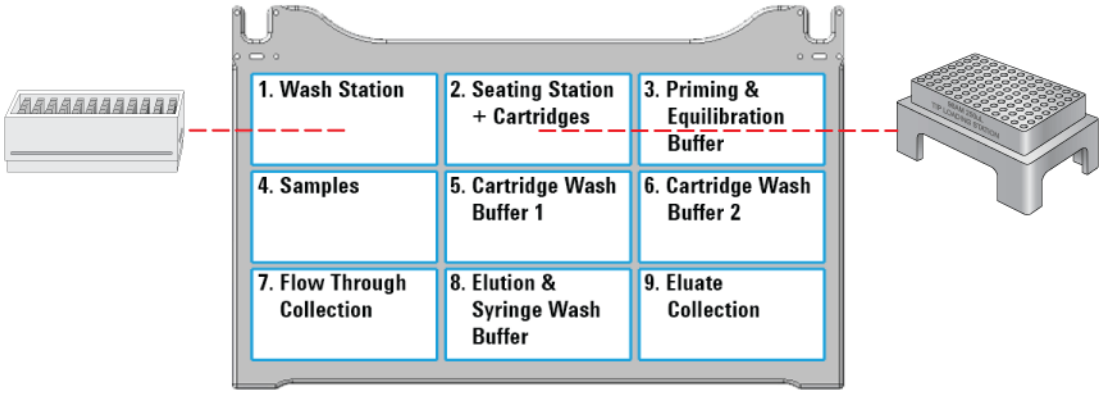
The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.

Note: The Greiner PCR plates are not compatible with the 25 µL cartridges at deck locations 7 and 9.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

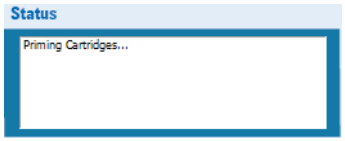
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

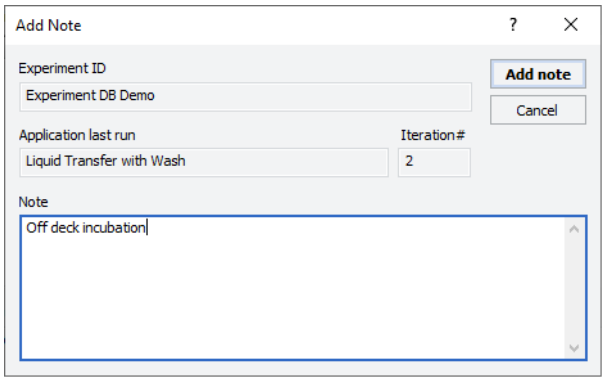
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

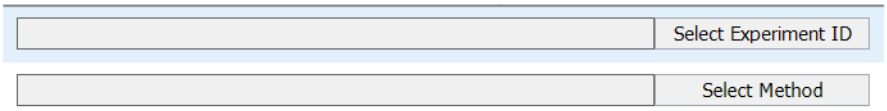
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click Run Protocol to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Affinity Purification protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12

Protocol step	Guidelines and notes
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the packed resin bed and properly wets the surface of the resin. In preparation for priming, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP affinity purification cartridges (PA-W, SA-W, and PG-W) typically used with this application contain affinity ligands (proteins) covalently immobilized onto resin. These cartridge types should be primed with aqueous buffers containing no or low amounts of organic solvent or known protein denaturants. Because the Equilibration Buffer is drawn from the same reservoir as the Priming Buffer, buffers that favor analyte binding with minimal non-specific binding should be used for both priming and equilibration. This step is selected by default. **Volume (µL)**. The default volume is sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default value diminishes the ability to effectively remove entrained air from the resin bed. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the sample loading begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates sample into the syringes, and then performs an external syringe wash at the wash station to remove any sample remaining on the outside of the probes before mounting the cartridges. The samples are dispensed through the cartridges into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any sample on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. The protocol accommodates sample volumes up to 1000 µL to be dispensed through the AssayMAP affinity purification cartridges. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. Each syringe has a maximum capacity of 250 µL. When sample volumes are greater than 250 µL, the protocol will iteratively load samples onto cartridges. To determine the number and volume of iterative load steps, the protocol uses the following formulas: • *# of times to load* = total sample volume/250, where # times to load is rounded up to nearest integer • *volume of each load* = sample volume/# of times to load For example, if the total sample volume is 900 µL, then: *# times to load* = 900/250 = 3.6, which is rounded up to 4 *volume of each load* = 900/4 = 225 If Collect Flow Through is selected for the Load Samples step, be sure that the Flow Through Collection plate has sufficient maximum well capacity. For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. **IMPORTANT** Be sure to include the recommended labware-specific volume overage to prevent air from entering the cartridge. For more information, see "Preparing the sample plates" on page 57. To determine the volume of sample to load, see "Determining the volume of sample to load" on page 56. This step is selected by default.

Protocol step	Guidelines and notes
	Volume (µL). The volume of sample to load should be balanced with the sample concentration and the mass capacity of the cartridge. • Default: 100 • Practical: 10–1000 • Range: 0–1000 *Note:* The lower the sample volume, the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min)**. The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than the default may not significantly increase analyte binding, and using flow rates faster than the default will decrease the quantitative binding capacity of the cartridges. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed into the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step is dispensed into the wash station. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is selected by default.

Protocol step	Guidelines and notes
Cup Wash 1	This step removes the residual sample solution that may remain above the resin bed after the Load Samples step. The Cup Wash 1 step aspirates Cartridge Wash Buffer 1 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the wash station, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 1	This step uses Cartridge Wash Buffer 1 to wash non-specifically bound molecules from the resin bed. In preparation for Internal Cartridge Wash 1, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Cartridge Wash Buffer 1 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 1 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. If the Load Samples step is selected, the first 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) of Cartridge Wash Buffer 1 is dispensed as a sample chase at the Load Samples flow rate. Next, the Internal Cartridge Wash 1 volume minus the chase volume is dispensed at the Internal Cartridge Wash 1 flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the flow-through from Internal Cartridge Wash 1 step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from Internal Cartridge Wash 1 is dispensed into the wash station. This step is not selected by default.
Cup Wash 2	This step removes the residual buffer that may remain above the resin bed after the Internal Cartridge Wash 1 step. This step aspirates Cartridge Wash Buffer 2 and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the previous cartridge wash is aspirated from the cartridge cups. Any cartridges that stuck to the probes during the cup wash are parked at the seating station, and then the liquid in the syringes is dispensed into the wash station. The syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. A volume less than the default may be insufficient for cup washing, while a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 2	This step uses Cartridge Wash Buffer 2 to wash non-specifically bound molecules and Cartridge Wash Buffer 1 from the resin bed. In preparation for Internal Cartridge Wash 2, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid that is in the cups is removed by a 60 µL aspiration, the aspirated solution is discarded at the wash station, 10 µL of Cartridge Wash Buffer 2 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 2 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges at the specified flow rate into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer from the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but will also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to 0 skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the liquid eluted during Internal Cartridge Wash 2 is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through is dispensed into the wash station. Select this step if you want to collect the flow-through generated during Internal Cartridge Wash 2. This step is not selected by default.
Stringent Syringe Wash	This step cleans the syringes with the Elution Buffer prior to elution. The Stringent Syringe Wash step aspirates the Elution Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the wash station. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume are unlikely to improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash cycle**. A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10
Elute	This step uses Elution Buffer to elute bound analytes from the cartridges. In preparation for elution, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Elution Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges, and then the cartridges are parked at the seating station. After the elution, the eluate is mixed in the Eluate Collection plate and the syringes are washed at the wash station. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples will not be mixed. You can also select the Eluate Discard and Add to Flow Through substeps, which are described in the following rows of this table.

Protocol step	Guidelines and notes
Elute (continued)	This step is selected by default. **Volume (µL)**. The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges, or 50–75 µL for 25 µL cartridges). The default volumes are conservative and significantly higher than the minimum expected with a strong Elution Buffer. *Note:* The Eluate Collection plate must be able to accommodate the total volume, which is determined by summing the net elution volume (Elute volume - Eluate Discard volume) and the Existing Collection Volume. For labware-specific maximum well volumes, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. • Volume for 5 µL cartridges: – Default: 25 – Practical: 10–30 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 125 – Practical: 50–150 – Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing. **Flow rate (µL/min).** A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more elution buffer may be required to get the same elution yield at high elution flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500 **Wash cycle**. The number of syringe washes to perform at the wash station after an Elute step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Eluate Discard	This substep of the Elute step permits a specified volume of the eluate from the cartridges to be discarded before the eluate starts to be collected during the Elute step. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the Elution Buffer at the Elute flow rate through the cartridges. If the Eluate Discard step is selected, the specified volume is dispensed into the wash station or Flow Through Collection plate (if the Add to Flow Through step is selected). The remaining Elution Buffer is dispensed through cartridges into the Eluate Collection plate. **Example**: If the Elute, Eluate Discard, and Add to Flow Through steps are all selected with the following settings: Elute volume = 15 µL (5 µL cartridges) or 40 µL (25 µL cartridges) Eluate Discard volume = 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) the first 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) eluate from the cartridges will be discarded into the Flow Through Collection plate, and the remaining 13 µL (5 µL cartridges) or 30 µL (25 µL cartridges) eluate will be collected in the Eluate Collection plate. Select the Eluate Discard step in situations where minimizing the volume of eluate is important. For AssayMAP cartridges, the initial elution volume (~2 µL for 5 µL cartridges and ~10 µL for the 25 µL cartridges) contains small or no measurable amounts of analyte. This option is not selected by default. **Volume (µL)**. The first volume of eluate that will be discarded during the Elute step. This value can equal, but cannot exceed the Elute volume. • Default: 0 • Practical: – 5 µL cartridges: 0–2 – 25 µL cartridges: 0–10 • Range: 0–250
Add to Flow Through	If selected, this step dispenses the Eluate Discard volume into the Flow Through Collection plate. If the Add to Flow Through step is not selected, the Eluate Discard is dispensed into the wash station. This step is not selected by default. *Note:* The Add to Flow Through step is an option only if both the Elute and Eluate Discard steps have been selected.

Protocol step	Guidelines and notes
Existing Collection Volume	This step enables you to specify an amount of liquid that is in the wells of the Eluate Collection plate at the beginning of the run. The Existing Collection Volume and the net volume from the Elute step (Elute volume - Eluate Discard volume) feeds into logic that adjusts the well-bottom offset for sample elution, calculates the eluate mixing volume, and dynamically moves the head into and out of the wells during elution and eluate mixing in a volume-dependent manner. For the maximum practical working volumes of labware for eluate collection, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. Select this step when the Eluate Collection plate contains a volume of liquid useful for immediately diluting the eluates, for adjusting the pH of the eluates, or to aid in the recovery of small volumes of eluates from AssayMAP cartridges. **Volume (µL):** • Default: 0 • Practical: 0—250 • Range: 0—1000 *Note:* Total elution collection well volumes above 500 µL may require additional off-deck mixing to reach homogeneity.
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, the 10 µL of elution buffer will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Affinity Purification protocol using the default method settings. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Start protocol	2	Parks any cartridges that may have been mounted on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes.
Prime	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then does an external probe wash.
	3	Aspirates 10 µL of Priming Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	3	Aspirates the Priming Buffer.
	2	Mounts the cartridges on the head.
	1	Dispenses the Priming Buffer through the cartridges into the wash station between the chimneys, and then does an external cartridge wash.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then does an external probe wash.
	3	Aspirates 10 µL of Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Equilibration Buffer into the cartridge cups and exercises the cartridges off task.
	3	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	1	Dispenses the Equilibration Buffer through the cartridges to equilibrate, and then does an external cartridge wash.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes at the wash station.
Load Samples	4	Aspirates samples into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	7	Dispenses the samples through the cartridges and into the Flow Through Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash 1	5	Aspirates Cartridge Wash Buffer 1 into the syringes.
	2	Washes the cartridge cups and exercises the cartridges off task.
	1	Dispenses the buffer into the wash station between the chimneys.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash 1	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then does and external probe wash.
	5	Aspirates 10 µL of Cartridge Wash Buffer 1 for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	5	Aspirates Cartridge Wash Buffer 1 into the syringes for the sample chase and Internal Cartridge Wash 1 steps.
	2	Mounts the cartridges on the head.
	1	Dispenses 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) Cartridge Wash Buffer 1 through the cartridges at the Load Samples flow rate for the sample chase step.
	1	Dispenses the remaining Cartridge Wash Buffer 1 through the cartridges at the Internal Cartridge Wash 1 flow rate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash 2	6	Aspirates Cartridge Wash Buffer 2 into the syringes.
	2	Washes the cartridge cups and exercises the cartridges off task.
	1	Dispenses buffer into the wash station between the chimneys.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash 2	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then does an external probe wash.
	6	Aspirates 10 µL of Cartridge Wash Buffer 2 for the cartridge air-gap prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates Cartridge Wash Buffer 2 into the syringes.
	2	Mounts the cartridges on the head.
	1	Dispenses Cartridge Wash Buffer 2 through the cartridges.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Stringent Syringe Wash	8	Aspirates the Stringent Syringe Wash Buffer (Elution Buffer).
	1	Dispenses the buffer at the wash station.
	1	Washes the syringes.
Elute	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then does an external probe wash.
	8	Aspirates 10 µL of Elution Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Elution Buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Elution Buffer.
	2	Mounts the cartridges on the head.
	9	Elutes the samples into the Eluate Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges at the seating station.
	9	Mixes eluates.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Final Syringe Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys.
	1	Washes the syringes.

Reference library

1 Terry, M.P. in Handbook of Affinity Chromatography, Second Edition. (ed. D.S. Hage), 367-397 (CRC Press, 2005).

2 PROTEUS Protein G Antibody Purification Handbook, Mini & Midi spin columns, Bio-Rad Laboratories, Inc 2013.

See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Bravo Platform.

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4 Affinity Purification: Aspiration Mode v3.0 User Guide

This chapter contains the following topics:

Note: This section presents instructions for using the Affinity Purification: Aspiration Mode v3.0 application. If you are using the Dispense Mode version, see "Affinity Purification v3.0 User Guide" on page 45.

App description

Affinity Purification: Aspiration Mode v3.0. This application enables automated affinity purification of target molecules, such as antibodies and peptides, from 1 to 96 samples in a single run. This application aspirates the sample and wash solutions up through the cartridge resin bed rather than dispensing them through the resin bed, which is how the standard Affinity Purification application functions.

For most customers, Agilent recommends using the standard Affinity Purification application instead of the Affinity Purification: Aspiration Mode application. Although the two applications yield similar results, the standard Affinity Purification application is less sensitive to clogging and, therefore, more robust. However, some customers find the Affinity Purification: Aspiration Mode application provides slightly better purification.

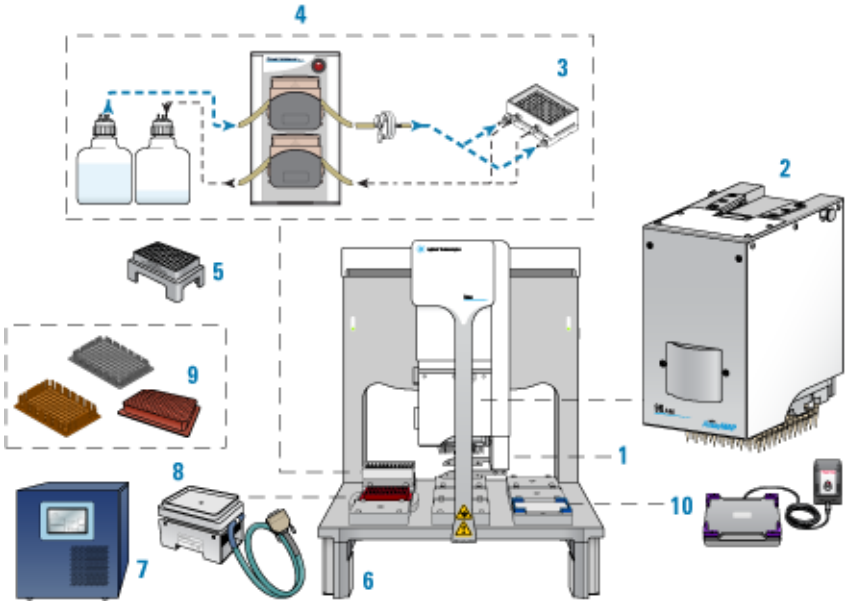
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Affinity Purification: Aspiration Mode protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Note: The 25-µL cartridges have not yet been optimized on the Affinity Purification: Aspiration Mode application. If you are interested in trying them on this application, contact Agilent Customer Service for advice.

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Affinity Purification on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Note: This application has not yet been optimized for the 25 µL cartridges.

Cartridge type	Agilent part number
AssayMAP Protein A (PA-W) cartridge rack	G5496-60000
AssayMAP Protein G (PG-W) cartridge rack	G5496-60008
AssayMAP Streptavidin (SA-W) cartridge rack	G5496-60010
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of Protein A, Protein G, or Streptavidin cartridges are required. For details, see Preparing the sample plates.	G5496-60009

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.
- PA-W, SA-W, and PG-W cartridges tolerate brief exposure to pH as low as 2.0. The stability of the PA-W, SA-W, and PG-W cartridges after capturing additional affinity ligands should be determined empirically.

Labware

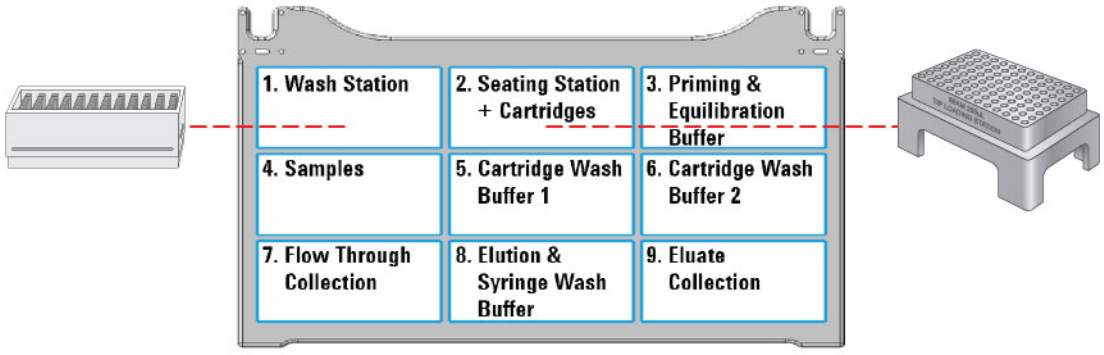
Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3—9
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3—9
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	3—9
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 675801, Half Area, Flat Bottom, UV Star	Greiner 675801	3—9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	3—9

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Reagents

The volume, type, and concentration of reagents required for affinity purification vary depending on sample characteristics and the desired analytical result. Consult the resources that cover general principles of affinity purification such as those listed in the "Reference library" on page 124. For examples of reagents used with specific affinity interactions, consult the published scientific literature including publications that use the AssayMAP Bravo which can be found in the Agilent AssayMAP Bravo Citation Index.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure due to the buildup of salts within the syringe barrels. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see "Syringe Wash v3.0 User Guide" on page 567.

All labware require volume overage for the protocol to execute properly. Use the Affinity Purification Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 95.

Preparing the solutions

The following solutions are required for the Affinity Purification: Aspiration Mode protocol:

- Priming & Equilibration Buffer
- Cartridge Wash Buffers
- Elution & Stringent Syringe Wash Buffer

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Affinity Purification: Aspiration Mode

The Reagent Volume Calculator is a Microsoft Excel file that contains the following:

- *Calculator worksheet*. You enter the number of columns to process, whether to perform the Collect Flow Through option, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

  Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.
- *Reagent Recipe worksheet*. You enter the concentrations of each component in your reagent, and the worksheet calculates the recipe volumes required.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click **Restore Defaults**.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figures show the worksheets of the Reagent Volume Calculator.

Figure Affinity Purification: Aspiration Mode Calculator worksheet

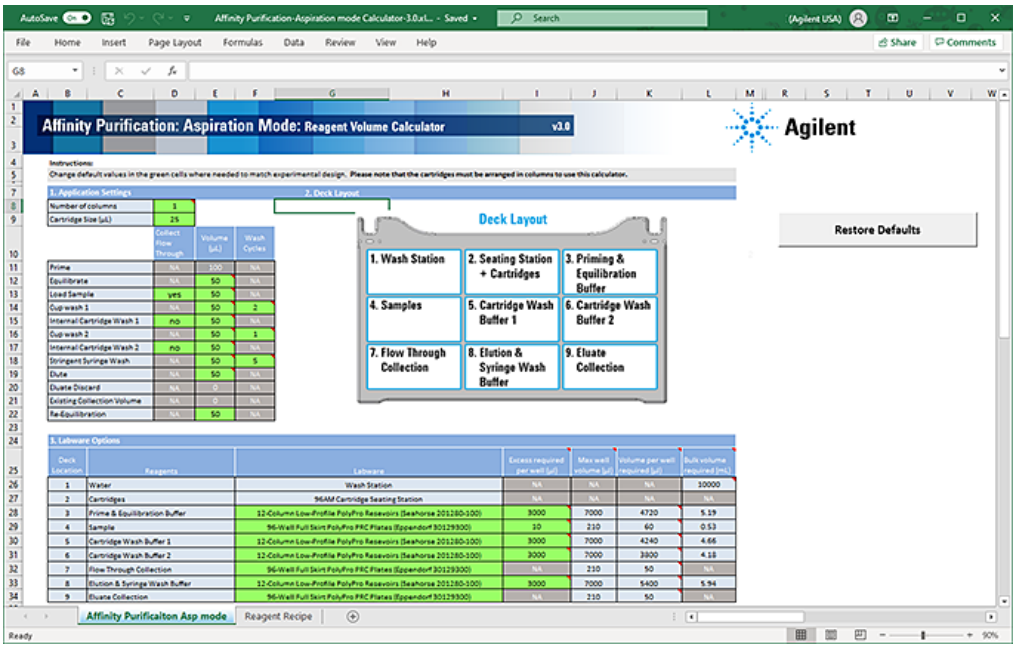
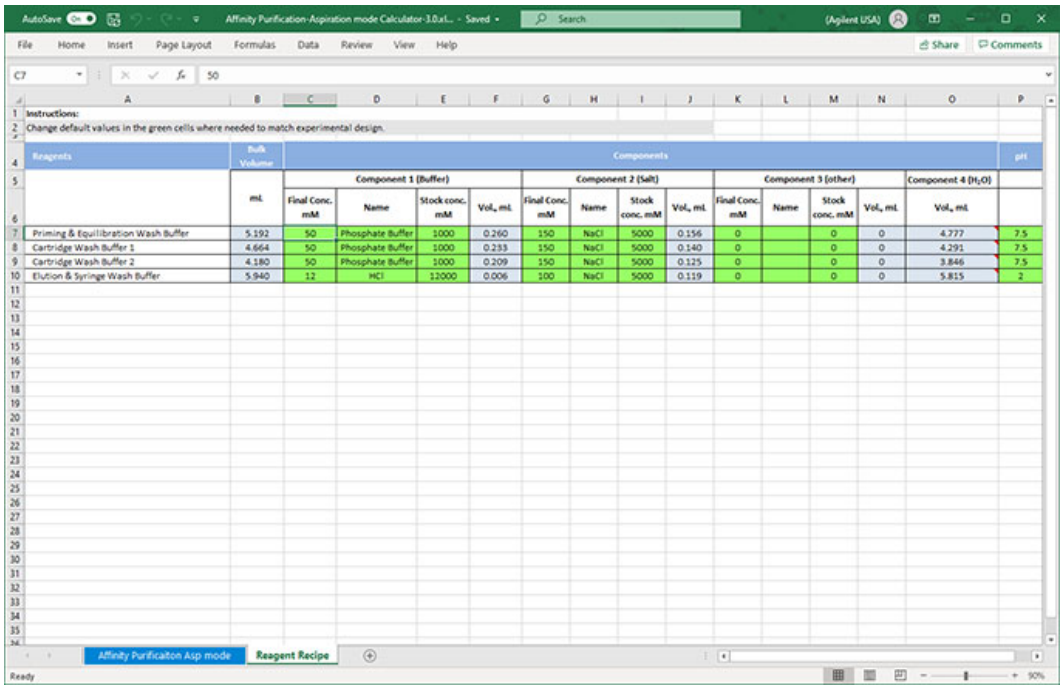


Figure Reagent Recipe worksheet



Preparing the buffers

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the AssayMAP affinity purification chemistry.

Table Reagent preparation

Reagent and deck location	Composition and comments
Priming & Equilibration Buffer (deck location 3)	Typically a buffered aqueous solution with neutral pH and physiologic salt concentration, such as Phosphate-Buffered Saline (PBS), that is similar in composition to the buffer solution used to prepare the sample.
Cartridge Wash Buffer 1 (deck location 5)	High-stringency buffer (for example, a neutral phosphate buffer with high NaCl) or a low-stringency buffer (for example, PBS or a neutral mass-spec-friendly buffer) The Affinity Purification: Aspiration Mode application is designed to allow the use of two wash buffers for high- and low-stringency washes or a single wash buffer. The buffer selection depends on a number of factors. First, consider whether Cartridge Wash Buffer 2 will or will not be used. This decision is often dependent on whether or not a stringent wash is desired in the protocol. If so, one would typically use the stringent buffer for Cartridge Wash Buffer 1 and then a less stringent wash buffer for Cartridge Wash Buffer 2, which would remove the stringent component of the buffer before elution. If a stringent buffer is not used, you would typically use Cartridge Wash Buffer 1 only, and it would be a low-stringency wash buffer, such as PBS or a neutral mass-spec-friendly buffer. *Note:* If you only do a single internal cartridge wash, it should be wash buffer 1, as skipping wash 1 and using wash 2 will result in skipping the sample chase which might decrease the amount of target recovered.
Cartridge Wash Buffer 2 (deck location 6)	Typically, a low-stringency buffer (for example PBS or a neutral mass-spec-friendly buffer) The composition of this buffer is often dictated by sensitivity of downstream steps to components of Cartridge Wash Buffer 2 as the void volume in the cartridges (~2 µL for 5 µL cartridges or 10 µL for 25 µL cartridges) will contain the last wash solution used before the Eluate step and end up in the eluate unless the Discard Eluate option is selected and this volume is discarded.
Elution & Syringe Wash Buffer (deck location 8)	12 mM HCl with 100 mM NaCl pH 2.0 or 5% acetic acid The elution solution is typically a low pH solution. A key consideration is if the sample will be neutralized following elution. If so, a 12 mM HCl solution with 100 mM NaCl is a good choice as it is very easy to neutralize. However, this solution approximately 4—6 column volumes to elute antibodies off the cartridge. If neutralization is not a key consideration or minimizing elution volume is a critical driver, a 5% acetic acid solution is a good choice because targets can be eluted in as little as 2 column volumes.

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and cartridge positions on the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 98.

To dispense the reagents into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Priming & Equilibration Buffer into the labware to be placed at deck location 3.
3. Add the specified volume of Cartridge Wash Buffer 1 into the labware to be placed at deck location 5.
4. If using two wash buffers, add the specified volume of Cartridge Wash Buffer 2 into the labware to be placed at deck location 6.
5. Add the specified volume of Elution & Syringe Wash Buffer into the labware to be placed at deck location 8.
6. If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT To minimize evaporation, prepare the samples immediately before running the Affinity Purification: Aspiration Mode protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to optimize the binding conditions (for example, pH).
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the sample composition

The optimal chemical environment for binding is generally similar for protein A, protein G, and streptavidin.

- Protein A and G resins bind selectively to antibodies.

  Examine the scientific literature for differences in their affinity for certain antibody subtypes from different species.
- Streptavidin resin binds selectively to biotinylated molecules.
- Protein A, protein G, and streptavidin are relatively unaffected by most sample components, including those present in complex protein mixtures.

What are optimal pH conditions?

One of the most important considerations for optimizing binding conditions is the pH of the sample, which should be near neutral pH. Both low (2 to 3) and high (10 to 11) pH ranges can prevent binding to protein A or protein G resins. In general, the sample should be:

- *Protein A*. Greater than pH 6.
- *Protein G*. Greater than pH 4.

What sample components cause concerns?

Protein A, protein G, and streptavidin generally tolerate moderate levels of salt, non-ionic detergents, and mild denaturing reagents, such as urea, quite well. You should examine scientific literature for the known effects and tolerances of protein A, protein G, and streptavidin, keeping in mind that these tolerances may differ depending on the antibody species and subtype.

Does the antibody species and isotype in the sample match the cartridge binding specificity?

Protein A and protein G bind a wide variety of antibody subtypes and species (1). Carefully consider the species and subtype of antibody when choosing between using the AssayMAP Protein A or Protein G cartridge for purification.

The antibodies that bind to protein G largely overlap the set that binds to protein A. While protein A is the industry standard for purification and titer determination of human therapeutic antibodies, protein G is the standard for purification of antibodies used as bioanalytical tools, primarily because many of these antibody subtypes are generated in species that bind poorly to protein A, for example, mouse IgG_1 and rat IgG_1 (2).

Determining the volume of sample to load

The AssayMAP Affinity Purification: Aspiration Mode protocol permits loading up to 1000 µL of sample onto AssayMAP cartridges. For sample volumes > 250 µL, the protocol will iteratively load samples onto cartridges to stay within the maximum syringe volume (250 µL) of the Bravo 96AM Head.

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of the target molecule that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through. This value is dependent on the sample load flow rate.
- *Total binding capacity*. The maximum mass of the target molecule that can bind to the cartridge. This can only be achieved by loading significantly more of the target molecule than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity.

See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed information about the binding capacity for Protein A, Protein G, and Streptavidin cartridges.

What is the concentration of the target in the sample?

If you know the approximate concentration of the target molecule in your sample and you are working within the quantitative binding range of the cartridge, you can determine the volume of sample to load as follows:

$$\text{µL sample to load} = \frac{\text{µg target desired}}{\text{µg/µL target in sample}}$$

Does the experiment require quantitative binding of the target?

For quantitative recovery of the target, the volume loaded must contain a mass of target protein that is equal to or less than the quantitative binding capacity. A bioprocess feed stream containing 10 mg/mL (10 µg /µL) antibody would require a maximum load volume of 10 µL (100 µg) to be within the quantitative range for 5 µL PA-W cartridges. If the antibody concentration is unknown, you can do multiple runs at different volumes (10, 100, and 1000 µL) to find a sample volume that is within the quantitative binding capacity, or a single run with the undiluted sample and multiple dilutions (no dilution, 1:10, and 1:100).

Preparing the sample plates

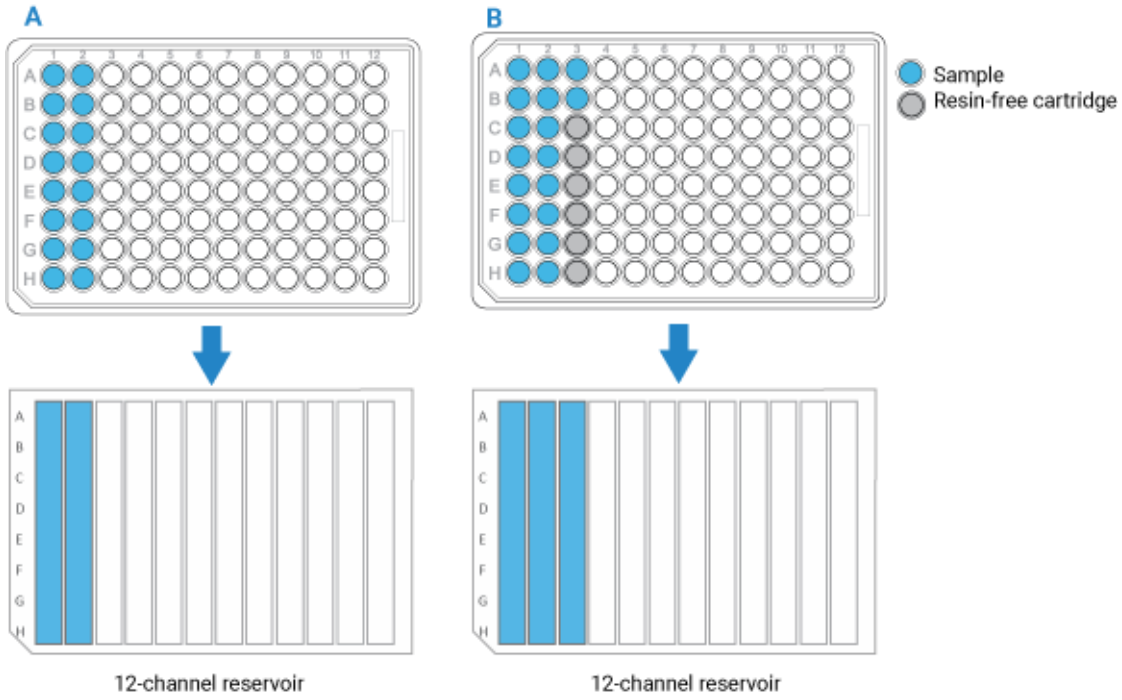
Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.

  The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based on the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use AssayMAP Resin-Free cartridges to fill the well positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash steps.

Figure Example of sample microplate and reservoir layout: A) Multiple of 8 samples, and B) Not a multiple of 8



See "Labware" on page 93 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION

A small volume excess is required in all labware types to ensure proper volume transfer.

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Affinity Purification: Aspiration Mode protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the analyte from the cartridges.

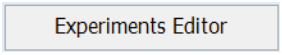
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the Cartridge & Tip Seating Station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 95.
- Prepare the samples. See "Preparing the samples" on page 98.

- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

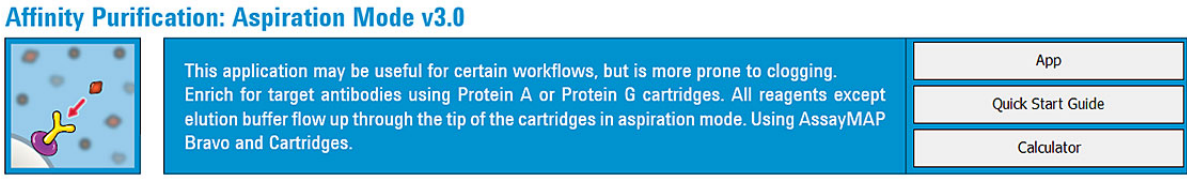
IMPORTANT Do not allow wetted cartridges to dry out. Agilent does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 93.

Setting up the protocol

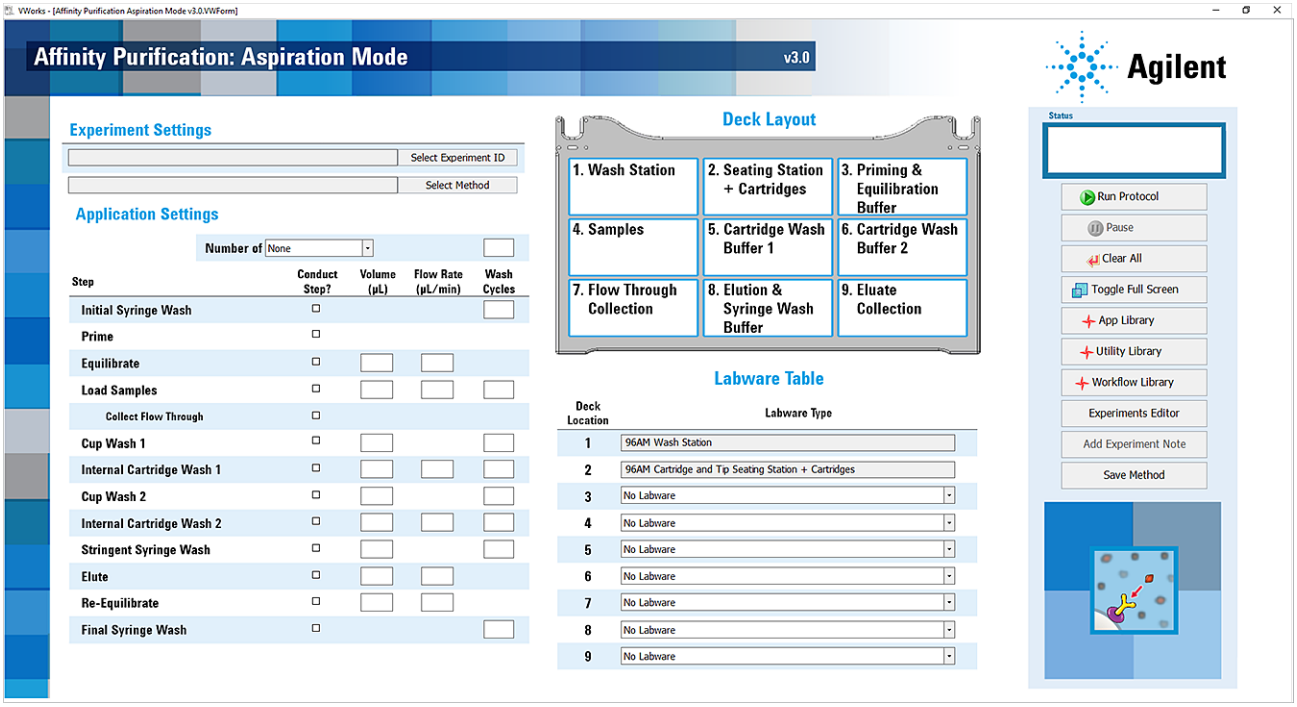
Before starting the protocol, make sure the appropriate selections and values are specified in the Affinity Purification: Aspiration Mode application.

To set up the protocol:

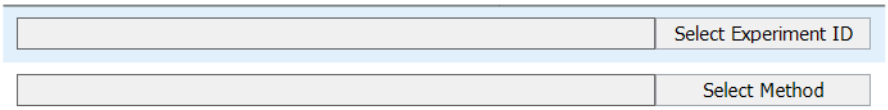
1. Open the **App Library**.
2. Locate **Affinity Purification: Aspiration Mode**, and then click **App**.



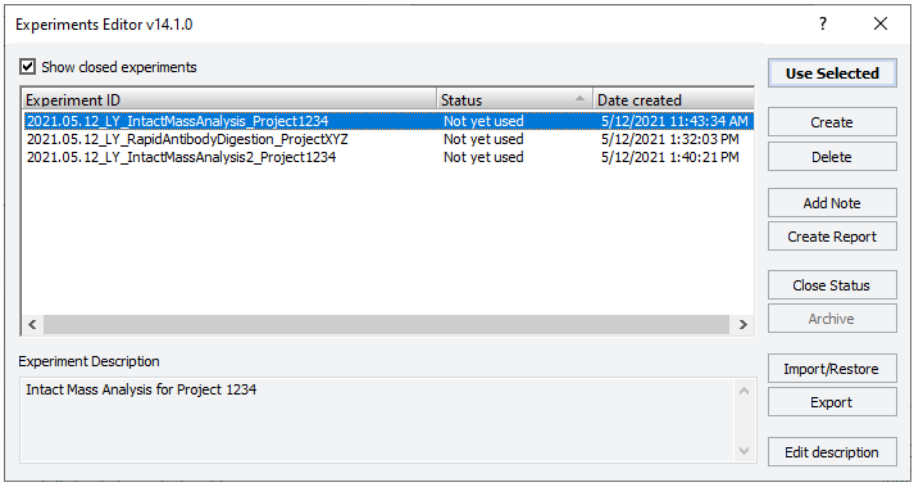
The Affinity Purification: Aspiration Mode application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 108.
- To create or modify a method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:

a Verify that the **Number of** box selection is **5µL Cartridges**.

b In the box, type the number of cartridges present in the cartridge holder at deck location 2. The position of the cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 8–96

Default: 8

IMPORTANT Ensure that you specify the **number of cartridges** for the Affinity Purification: Aspiration Mode app instead of the number of columns, which is the setting used in the other AssayMAP apps. Also, make sure to use a column format for the microplate setup to avoid droplets falling on the deck as described in "Planning the microplate setup" on page 100.

CAUTION **If the cartridge selection is greater than the actual number of cartridges used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the form specifies 96 cartridges, but only 8 cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.**

CAUTION **If the cartridge selection is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the form specifies 8 cartridges, but 96 cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. To prevent potential equipment damage, ensure that the column selection is correct.**

IMPORTANT Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings** area, select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 110.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates the Priming & Equilibration buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	100	300	–
		Range:	100	300	–
Equilibrate	Dispenses the Priming & Equilibration buffer through the cartridges into the wash station (deck location 1).	5 µL:	50	10	–
		Range:	0–140	0.5–500	–
Load Samples	Aspirates up to 245 µL of samples (deck location 4) through the mounted cartridges into the syringes, performs an external cartridge tip wash at the wash station (deck location 1), and then aspirates a 5- µL chase of Equilibration Buffer (deck location 3). The cartridges are removed (deck location 2) and then the flow-through is dispensed into either Flow Through Collection (deck location 7) or the wash station (deck location 1). Samples >245 µL are loaded in multiple steps.	5 µL:	100	5	3
		Range:	0–1000	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through at the Flow Through Collection (deck location 7). If not selected, discards the sample flow-through at the wash station (deck location 1).	–	–	–	–
Cup Wash 1	Rinses the cartridge cups with Cartridge Wash Buffer 1 (deck location 5), and then discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		Range:	0–100	–	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Internal Cartridge Wash 1	Aspirates Cartridge Wash Buffer 1 (deck location 5) through the mounted cartridges, removes the cartridges from the probes, and dispenses the contents of the syringes into the wash station (deck location 1).	5 µL:	50	10	3
		Range:	0–250	0.5–500	0–10
Cup Wash 2	Rinses the cartridge cups with Cartridge Wash Buffer 2 (deck location 6) and discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 2	Aspirates Cartridge Wash Buffer 2 (deck location 6) through the mounted cartridges, removes the cartridges from the probes, and dispenses the contents of the syringes into the wash station (deck location 1).	5 µL:	50	10	3
		Range:	0–250	0.5–500	0–10
Stringent Syringe Wash	Aspirates Syringe Wash Buffer (deck location 8) into the syringes, and then discards the liquid into the wash station (deck location 1).	5 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates Elution Buffer (deck location 8) into the syringes, and then dispenses the buffer through the cartridges into the Eluate Collection plate (deck location 9).	5 µL:	25	5	–
		Range:	0–250	0.1–500	–
Re-Equilibrate	Aspirates Equilibration Buffer (deck location 3) through the mounted cartridges into the syringes (aspirate mode), removes the cartridges, and then dispenses liquid into the wash station (deck location 1).	5 µL:	50	10	–
		Range:	0–250	0.5–500	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 111.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 121.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT The protocol will display an error message if cartridges are missing.

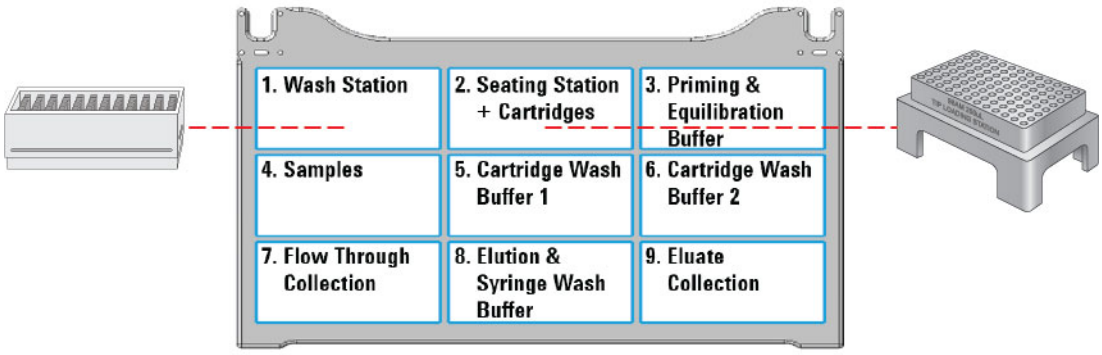
Starting the protocol run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

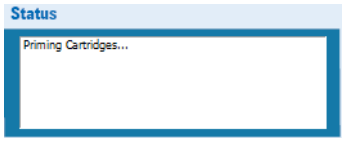
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

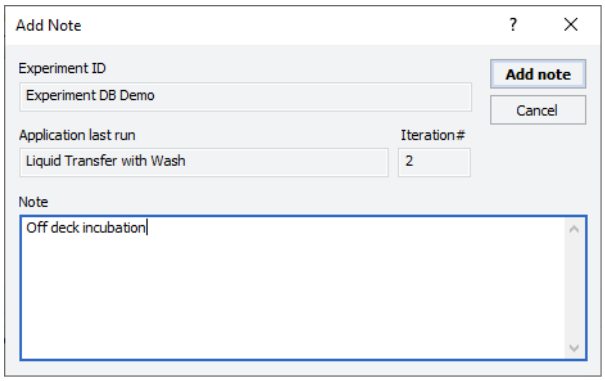
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



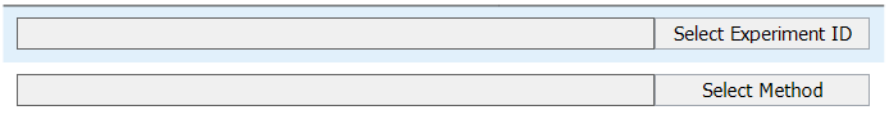
2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

1 Remove used labware from the deck.
2 Discard leftover reagents appropriately.
3 *Optional*. Conduct stringent washing of the syringes:
   a Open the **Syringe Wash** utility  .
   b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Affinity Purification: Aspiration Mode protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that this selection is correct. If the cartridge selection is: • *Greater than the actual number used,* the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the form specifies 96 cartridges, but only 8 cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 8 cartridges, but 96 cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. **IMPORTANT** Although you specify the number of cartridges for this app, you specify the number of columns of cartridges for other apps. Default: 8 Range: 1-12
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the packed resin bed and properly wets the surface of the resin. The Prime step aspirates enough Priming Buffer into the syringes to wet the inlet seal (10 µL), and perform the Prime (100 µL) and Equilibration (50 µL default) steps. In preparation for priming, 10 µL of Priming Buffer is dispensed into each cartridge cup to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. After mounting the cartridges on the probes, the Priming Buffer is dispensed (100 µL at 300 µL/min) through the cartridges into the wash station. The AssayMAP affinity purification cartridges (PA-W, SA-W, and PG-W) typically used with this application contain proteins covalently immobilized onto resin. These cartridge types should be primed with aqueous buffers containing no or low amounts of organic solvent or known protein denaturants. Because the Equilibration Buffer is drawn from the same reservoir as the Priming Buffer, buffers that favor analyte binding should be used for both priming and equilibration. This step is selected by default. **Volume (µL)**. The volume is sufficient to wet and remove entrained air from the resin bed. • Default: 100 • Range: 100 **Flow rate (µL/min)**. • Default: 300 • Range: 300
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. This step dispenses the Equilibration Buffer remaining in the syringes through the cartridges. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Default: 50 • Practical: 50–100 • Range: 0–140 If you select Prime and Equilibrate, the system calculates the volume required to prewet the cartridges and perform both steps and then aspirates that volume during the Prime step. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. This step mounts the cartridges on the syringe probes, aspirates the sample through the resin bed, and washes the exterior of the cartridge tips at the wash station to remove any sample on the outside of the cartridge tips. A 5 µL sample chase of Priming & Equilibration Buffer is aspirated through the resin bed at the same flow rate as the sample load to ensure that the sample in the resin bed at the end of the sample load has the same residence time for binding as the rest of the sample. An exterior cartridge wash is performed, cartridges are ejected, and then the contents in the syringe (flow-through) are deposited into the Flow Through Collection plate or the wash station. The protocol accommodates sample volumes up to 1000 µL to be aspirated through the AssayMAP affinity purification cartridges. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. Each syringe has a maximum capacity of 250 µL. When sample volumes are greater than 250 µL, the protocol will iteratively load samples onto cartridges in equal volume steps. To determine the number of times to load and the volume of each iterative load, the protocol uses the following formulas: *# of times to load* = total sample volume/250, where # times to load is rounded up to nearest integer *volume of each load* = sample volume/# of times to load For example, if the total sample volume is 900 µL, then: *# times to load* = 900/250 = 3.6, which is rounded up to 4 *volume of each load* = 900/4 = 225 If Collect Flow Through is selected for the Load Samples step, be sure that the Flow Through Collection plate has sufficient maximum well capacity. For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. **IMPORTANT** Be sure to include the recommended labware-dependent volume overage to prevent air from entering the cartridge. For more information, see "Preparing the sample plates" on page 100. This step is selected by default. **Volume (µL).** The volume of sample should be balanced with the sample concentration and the mass capacity of the cartridge. To determine the volume of sample to load, see "Determining the volume of sample to load" on page 99. • Default: 100 • Practical: 10–1000 • Range: 0–1000 *Note:* The lower the sample volume the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing.

Protocol step	Guidelines and notes
	Flow rate (µL/min). The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than the default may not significantly increase analyte binding, and using flow rates faster than the default will decrease the quantitative binding capacity of the cartridges. • Default: 5 • Practical: 2–15 • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed into the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step is dispensed into the wash station. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is not selected by default. *Note:* A constant 5-µL volume is used as a sample chase during the Load Samples step, which results in some dilution of sample flow-through. For example, if you load a 50-µL sample, the flow-through will contain the volume of the sample plus 5 µL of sample chase. Make sure you consider this volume when calculating the flow-through concentration.

Protocol step	Guidelines and notes
Cup Wash 1	This step removes the residual sample solution that may remain above the resin bed after the Load Samples step. The Cup Wash 1 step aspirates Cartridge Wash Buffer 1 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the wash station, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 1	This step uses Cartridge Wash Buffer 1 to wash non-specifically bound molecules from the resin bed. For the wash operation, this step aspirates Cartridge Wash Buffer 1 through the mounted cartridges into the syringes, parks the cartridges at the seating station, and then dispenses the syringe contents into the wash station at an offset from the chimneys. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Cup Wash 2	This step removes the residual buffer that may remain above the resin bed after the Internal Cartridge Wash 1 step. This step aspirates Cartridge Wash Buffer 2 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the previous cartridge wash is aspirated from the cartridge cups. Any cartridges that stuck to the probes during the cup wash are parked at the seating station, and then the liquid in the syringes is dispensed into the wash station. The syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. A volume less than the default may be insufficient for cup washing, while a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 2	This step uses Cartridge Wash Buffer 2 to wash non-specifically bound molecules and Cartridge Wash Buffer 1 from the resin bed. For the wash operation, this step aspirates Cartridge Wash Buffer 2 through the mounted cartridges into the syringes, parks the cartridges at the seating station, and then dispenses the buffer into the wash station at an offset from the chimneys. The syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but will also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to 0 skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Stringent Syringe Wash	This step cleans the syringes with the Elution Buffer prior to elution. The Stringent Syringe Wash step aspirates the Elution Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the wash station. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL/min)**. Volumes higher than the default volume may improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash cycle**. A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10
Elute	This step uses Elution Buffer to elute bound analytes from the cartridges. This step aspirates the buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges. This step is selected by default. **Volume (µL).** The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges). The default volume is conservative and significantly higher than the minimum expected with a strong Elution Buffer. • Default: 25 • Practical: 10–30 • Range: 0–250 **Flow rate (µL/min).** A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more elution buffer may be required to get the same elution yield at high elution flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500

Protocol step	Guidelines and notes
Re-Equilibrate	Uses the Equilibration Buffer to return the cartridge to a neutral pH solution. During the Re-Equilibrate step, the Equilibration Buffer is aspirated through the mounted cartridges into the syringes. The cartridges are parked at the seating station and then the liquid is dispensed into the wash station between the chimneys. The syringes are washed, and then the syringes are used to mix the samples in the Eluate Collection plate. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples are not mixed. This step is selected by default. *Note:* If the Re-Equilibrate step is skipped, the eluate will not be mixed. **Volume (µL).** The default volume is equal to 10-column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. During each Final Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Minimizing the duration of your assay

To minimize the duration of your assay:

If the sum of the sample load, chase, and both internal cartridge washes is less than 250 µL, modify the following protocol settings to minimize the Bravo Platform actions:

- **Load Samples**: Set the number of wash cycles to 0.
- **Collect Flow Through**: Not selected.
- **Cup Wash 1**: Not selected.
- **Internal Cartridge Wash 1:** Set the number of wash cycles to 0. (If Internal Cartridge Wash 2 is not selected, set the number of wash cycles to any desired value.)
- **Cup Wash 2**: Not selected.

These settings result in accumulation of the flow-through, chase, and any internal cartridge wash liquids in the syringe without requiring removal of the cartridges from the probes. This total volume must be less than the 250 µL syringe capacity, as the cartridges will dismount for larger accumulated volumes and introduce the potential for contamination of the cup, which would make skipping the cup washes a poor choice. The software keeps track of cumulative volume of the sample (which equals the flow-through), 5-µL chase, and Internal Cartridge Wash 1 and 2 drawn into the syringe, and performs the steps required to empty the syringe when it reaches the 250-µL capacity. As long as the accumulated volume is less than 250 µL, this volume can be held in the syringes until the Stringent Syringe Wash initiates.

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Affinity Purification: Aspiration Mode protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Start protocol	2	Parks any cartridges that may have been mounted on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes the specified number of times.
Prime	3	Aspirates the Priming & Equilibration Buffer for the air-gap prevention and the Prime and Equilibrate steps.
	2	Dispenses 10 µL of buffer into each cartridge cup for air-gap prevention.
	2	Mounts the cartridges on the syringe probes.
	1	Dispenses the buffer for the Prime step through the cartridges into the wash station at the priming flow rate.
Equilibrate	1	Dispenses the remaining buffer through the cartridges into the wash station at the Equilibration flow rate, and then washes the exterior of the cartridges.

Protocol step	Head moves to deck location...	Action
Load Samples	4	Aspirates samples through the mounted cartridges into the syringes.
	1	Washes the exterior of the cartridges.
	3	Aspirates a 5 µL of Priming & Equilibration Buffer for the sample chase.
	1	Washes the exterior of the cartridges.
	2	Parks the cartridges at the seating station.
	1	Dispenses the sample flow-through into the wash station.
	1	Washes the syringes.
Cup Wash 1	5	Aspirates Cartridge Wash Buffer 1 into the syringes.
	2	Performs the cup wash and exercises the cartridges off task.
	1	Dispenses the buffer at the wash station.
	1	Washes the syringes at the wash station.
Internal Cartridge Wash 1	2	Mounts the cartridges on the syringe probes.
	5	Aspirates Cartridge Wash Buffer 1 through the mounted cartridges.
	1	Washes the exterior of the cartridges.
	2	Parks the cartridges at the seating station.
	1	Dispenses the buffer into the wash station.
	1	Washes the syringes.
Cup Wash 2	6	Aspirates Cartridge Wash Buffer 2 into the syringes.
	2	Performs the cup wash and exercises the cartridges off task.
	1	Dispenses the buffer at the wash station.
	1	Washes the syringes at the wash station.
Internal Cartridge Wash 2	2	Mounts the cartridges on the syringe probes.
	6	Aspirates Cartridge Wash Buffer 2 through the mounted cartridges.
	1	Washes the exterior of the cartridges.
	2	Parks the cartridges at the seating station.
	1	Dispenses the buffer into the wash station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Stringent Syringe Wash	8	Aspirates Syringe Wash Buffer.
	1	Dispenses the buffer into the wash station.
	1	Washes the exterior of the syringes.
Elute	8	Aspirates the Elution Buffer.
	2	Mounts the cartridges on the syringe probes.
	9	Elutes the samples into the Eluate Collection plate.
	1	Washes the exterior of the cartridge tips.
Re-Equilibrate and Eluate Mix	3	Aspirates Equilibration Buffer through the cartridges into the syringes.
	2	Parks the cartridges at the seating station.
	1	Dispenses the buffer, and then washes the syringes.
	9	Mixes the samples in the Eluate Collection plate using the syringes.
Final Syringe Wash	1	Washes the syringes.

Reference library

1 Terry, M.P. in Handbook of Affinity Chromatography, Second Edition. (ed. D.S. Hage), 367-397 (CRC Press, 2005).

2 PROTEUS Protein G Antibody Purification Handbook, Mini & Midi spin columns, Bio-Rad Laboratories, Inc 2013.

5 Fractionation v2.0 User Guide

This chapter contains the following topics:

App description

Fractionation v2.0. This application enables automated stepwise elution of samples in up to six fractions using different stringency solutions, such as buffers with increasing ionic strength, increasing pH, or increasing organic concentration.

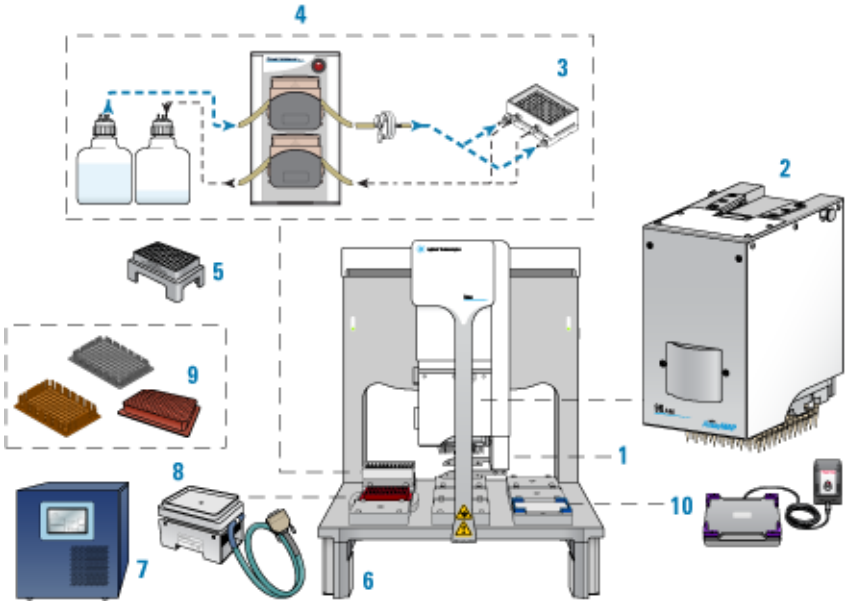
Before you start

This topic lists the required hardware, software, AssayMAP kits, cartridges, labware, and reagents for running the Fractionation protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge
- Microplate vacuum concentrator for concentration and drying of samples
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

Starter kit, cartridges, and labware

The starter kit for the Fractionation application contains both strong cation exchange (SCX) cartridges and labware.

Starter kit	Agilent Part number
AssayMAP SCX Fractionation Starter Kit— Contains 96 strong cation exchange (SCX) cartridges and Labware for the Fractionation protocol.*	G5496-60014
* The SCX Fractionation kit includes16x Greiner 650201_U-Bottom, Clear PolyPro and 1x 96 ABgene 1127, 1mL Deep Well, Square Well, Round bottom plate.	

AssayMAP cartridges

The following table lists the available AssayMAP cartridges that are commonly used to perform fractionation on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP SCX cartridge rack (Qty 96)	5190-6536	–
AssayMAP C18 cartridge rack (Qty 96)	5190-6532	G5496-60017
AssayMAP RP-S cartridge rack (Qty 96)	G5496-60033	G5496-60023

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of SCX, C18, or RP-S 5- or 25-µL cartridges are required. For details, see "Preparing the sample plates" on page 134.	G5496-60009	–

Note: The C18 and RP-S cartridges contain reversed-phase resin. Agilent suggests using RP-S for high pH reversed-phase fractionation because it is more stable in high pH solutions.

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.

Labware

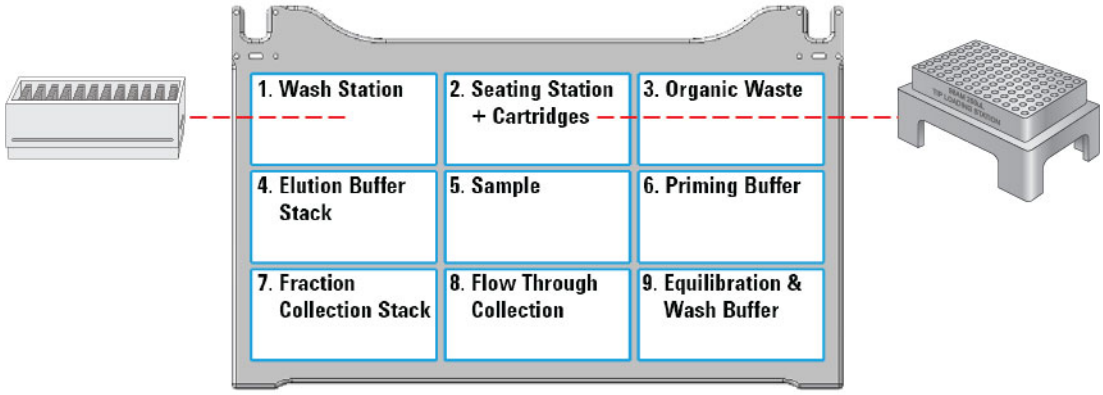
Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4—9
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	4—9

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Reagents

The AssayMAP Fractionation protocol is designed to permit multiple types of fractionation to be performed. Therefore, the reagent requirements are dependent on the fractionation strategy and the chemistry of the AssayMAP cartridges. For examples, consult the published scientific literature, including publications that use the AssayMAP Bravo which can be found in the Agilent AssayMAP Bravo Citation Index.

In general, buffer systems used for liquid chromatography separations in a column format can be adapted for use in the AssayMAP cartridge format. Fractionation using AssayMAP SCX cartridges can be accomplished using elution buffers with increasing ionic strength or increasing pH.

Similarly, both C18 and RP-S cartridges can be used for high-pH, reversed-phase fractionation using increasing concentrations of organic solvent in the elution buffers. Because of the high-pH stability of RP-S cartridges, RP-S cartridges are recommended for high-pH, reversed-phase fractionation with increasing concentrations of organic solvent.

All labware require volume overage for the protocol to execute properly. Use the Fractionation Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 131.

Preparing the solutions

The following solutions are required for the Fractionation protocol:

- Priming Buffer
- Equilibration and Wash Buffer
- Elution Buffers

You can use the supplied Reagent Volume Calculator for the Fractionation application to facilitate recipe volume calculations.

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Fractionation

The Reagent Volume Calculator is a Microsoft Excel file that contains a Calculator worksheet. You enter the number of columns to process, whether to perform the Collect Flow Through options, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

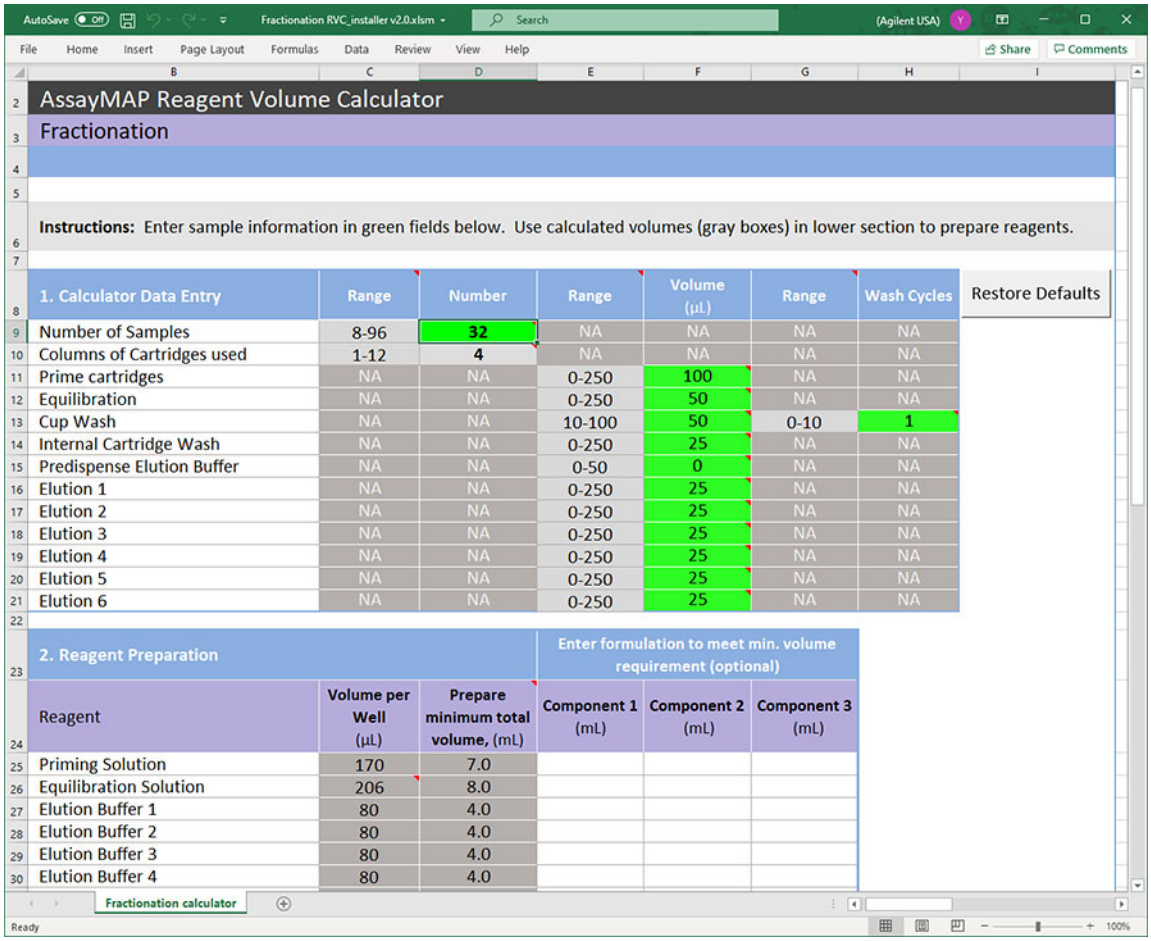
To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click **Restore Defaults** to set the values in the calculator using the values from the default method for the 5 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figure shows the worksheet of the Reagent Volume Calculator.



AssayMAP Reagent Volume Calculator

Fractionation

Instructions: Enter sample information in green fields below. Use calculated volumes (gray boxes) in lower section to prepare reagents.

1. Calculator Data Entry	Range	Number	Range	Volume (µL)	Range	Wash Cycles	Restore Defaults
Number of Samples	8-96	32	NA	NA	NA	NA	
Columns of Cartridges used	1-12	4	NA	NA	NA	NA	
Prime cartridges	NA	NA	0-250	100	NA	NA	
Equilibration	NA	NA	0-250	50	NA	NA	
Cup Wash	NA	NA	10-100	50	0-10	1	
Internal Cartridge Wash	NA	NA	0-250	25	NA	NA	
Predispense Elution Buffer	NA	NA	0-50	0	NA	NA	
Elution 1	NA	NA	0-250	25	NA	NA	
Elution 2	NA	NA	0-250	25	NA	NA	
Elution 3	NA	NA	0-250	25	NA	NA	
Elution 4	NA	NA	0-250	25	NA	NA	
Elution 5	NA	NA	0-250	25	NA	NA	
Elution 6	NA	NA	0-250	25	NA	NA	

2. Reagent Preparation			Enter formulation to meet min. volume requirement (optional)		
Reagent	Volume per Well (µL)	Prepare minimum total volume, (mL)	Component 1 (mL)	Component 2 (mL)	Component 3 (mL)
Priming Solution	170	7.0			
Equilibration Solution	206	8.0			
Elution Buffer 1	80	4.0			
Elution Buffer 2	80	4.0			
Elution Buffer 3	80	4.0			
Elution Buffer 4	80	4.0			

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and the cartridge positions in the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 133.

To dispense the reagents into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Priming Buffer into the labware to be placed at deck location 6.
3. Add the specified volume of Equilibration & Wash Buffer into the labware to be placed at deck location 9.

4 Add the specified volume of Elution Buffer into the labware (1–6) to be stacked at deck location 4.

5 If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT To minimize evaporation, prepare the samples immediately before running the Fractionation protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to optimize the binding conditions (for example, pH).
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the buffer composition

Depending on the type of fractionation you are performing the sample should be in a buffer compatible with the stationary phase. For peptide fractionation using SCX cartridges, optimum performance is typically achieved under low salt and low pH conditions. For high- or low-pH peptide fractionation using C18 and RP-S cartridges, samples typically should have a pH of < 3.

Determining the volume of sample to load

The AssayMAP Fractionation protocol permits loading up to 250 µL of sample onto AssayMAP cartridges due to labware capacity limits.

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of peptide that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through. For reversed-phase cartridges, the quantitative binding capacity is relatively straightforward for a single species of peptide. The quantitative binding capacity for a mixture of peptides is more complex due to the differences in relative hydrophobicity of the peptides, which results in competitive binding in situations where the ratio of binding sites to mass loaded is low.

  For examples of how sample mass loaded and the relative hydrophobicity of peptides can affect recovery in a complex peptide mixture, see Agilent Application Note 5991-2957EN in the ""Reference library" on page 158.

  To avoid or minimize sample bias during peptide cleanup, it is critical to load sample masses that are less than the mass at which hydrophillic peptides are lost. See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed quantitative cartridge binding capacity information for the 5 and 25 µL RP-S and C18 cartridges.
- *Total binding capacity*. The maximum mass of peptide that can bind to the cartridge. This can only be achieved by loading significantly more peptide than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity and will result in the loss of hydrophillic peptides. See the *Agilent AssayMAP Bravo Cartridges Selection Guide* for detailed total binding capacity.

Preparing the sample plates

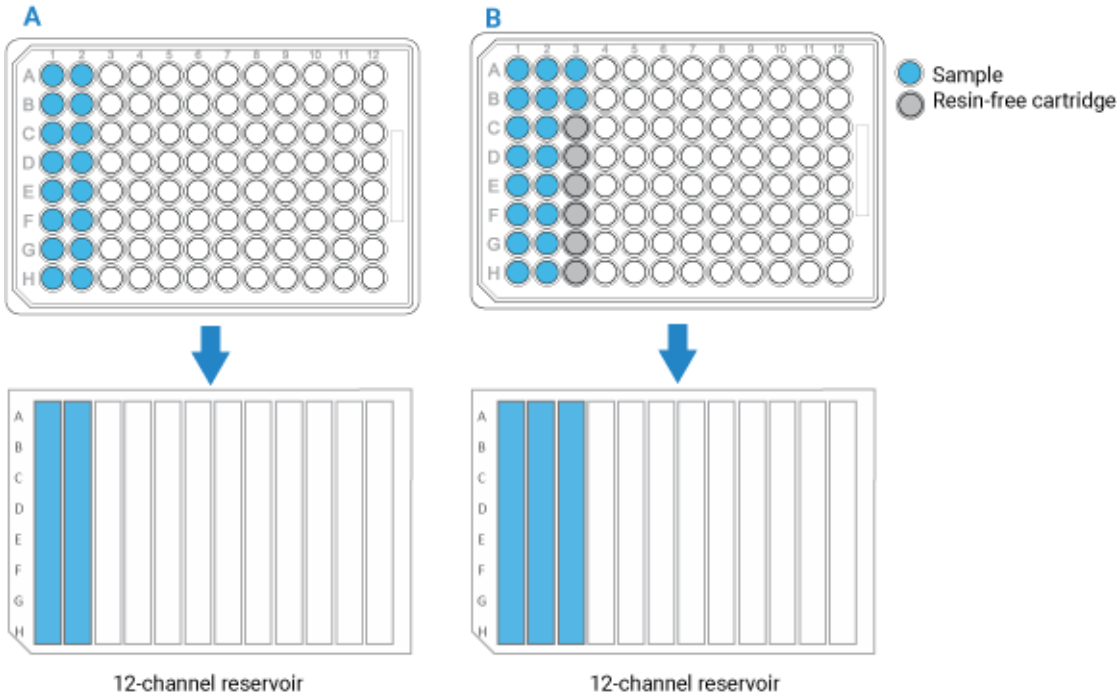
Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.

  The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based on the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use AssayMAP Resin-Free cartridges to fill the empty well positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash steps.

Figure Example of sample microplate and reservoir layout: A) Multiple of 8, and B) Not a multiple of 8



See "Labware" on page 129 for acceptable labware at each deck location.

Transferring the samples to the microplate

You can transfer the samples to the microplate that is supplied with the AssayMAP kits. See "Starter kit, cartridges, and labware" on page 128.

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer.**

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1 Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:

- "Reagent Transfer v3.0 User Guide" on page 525
- "Reformatting v3.0 User Guide" on page 623

2 If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Fractionation protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the peptides from the cartridges.

For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the 96AM Cartridge & Tip Seating Station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 131.
- Prepare the samples. See "Preparing the samples" on page 133.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See "System Startup/Shutdown v3.0 User Guide" on page 574.
- Transfer the cartridges to the Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

IMPORTANT Do not allow wetted cartridges to dry out. Agilent does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 129.

About stacking Fraction Collection plates and Elution Buffer plates

You must prepare stacks of pre-filled Elution Buffer plates and empty Fraction Collection plates on the Bravo deck before running the Fractionation protocol. You will use the Plate Stacking protocol in this app to stack the plates.

CAUTION **You should always use the Plate Stacking protocol to stack the plates. Stacking the labware manually can produce misaligned stacks, resulting in crashes during the protocol run.**

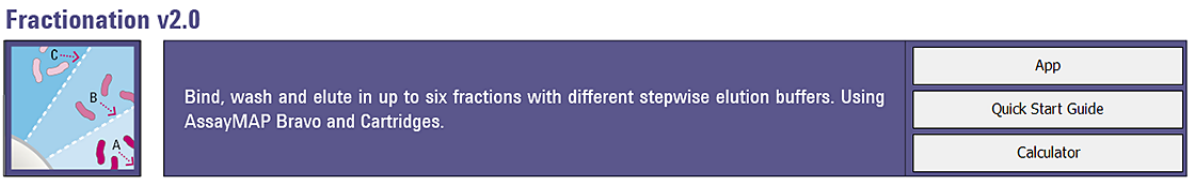
CAUTION **Do not use labware that have been repeatedly heat sealed or subjected to extreme heat or cold. Warped labware can cause imprecise stacking, potentially causing head crashes during the protocol run.**

Setting up the protocol

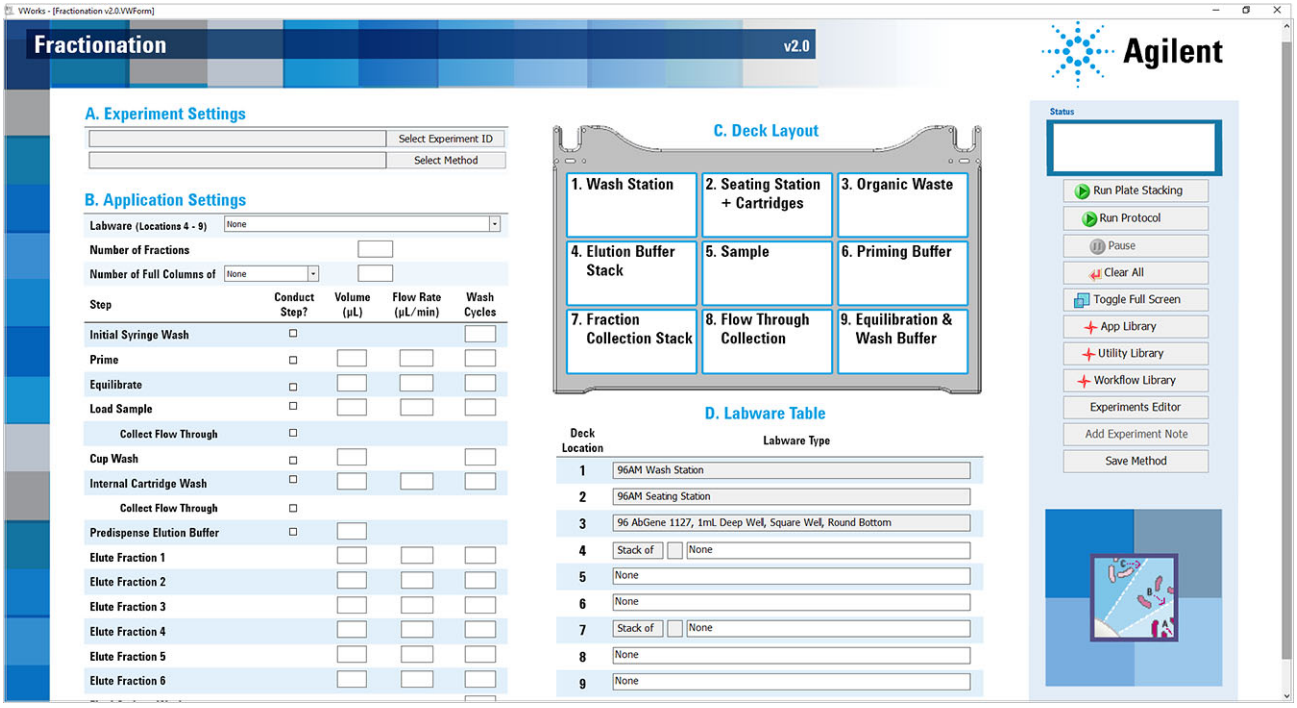
Before starting the protocol, make sure the appropriate selections and values are specified in the Fractionation application.

To set up the Plate Stacking and Fractionation protocols:

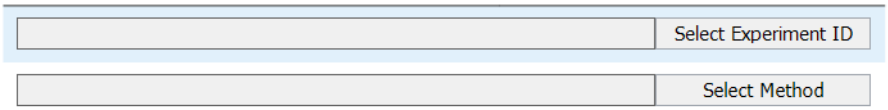
1. Open the **App Library**.
2. Locate **Fractionation**, and then click **App**.



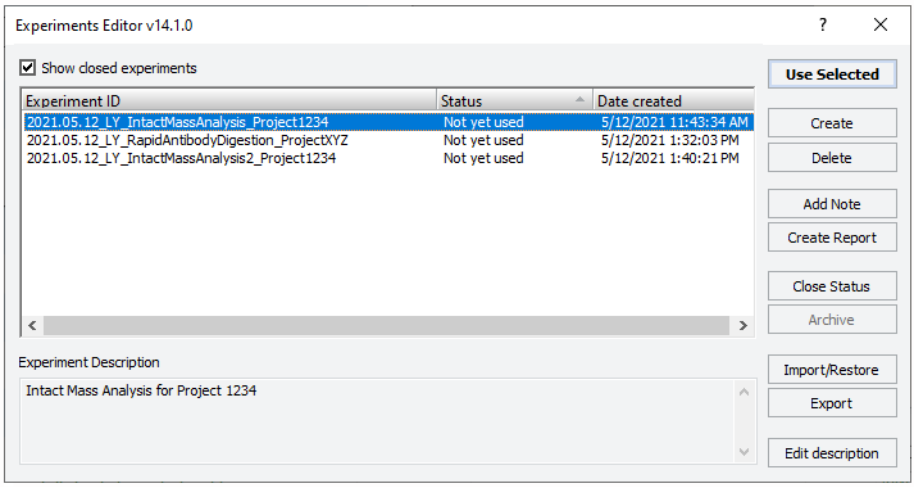
The Fractionation application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 142.
- To modify the method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create or modify methods.

6 In the **Application Settings** area, specify the following to create or modify a method:

a Select the labware you are using from the **Labware (Locations 4 - 9)** list.

b In the **Number of Fractions** box, type a value from 0 to 6 for the fractions that will be collected during the Fractionation protocol run. The value you specify must match the number Elution Buffer and collection plates you are using.

c Specify the cartridge settings:

- Select the cartridge size from the list:
  5 µL Cartridges or 25 µL Cartridges
- In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION **If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.**

CAUTION **If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.**

IMPORTANT Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 145.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 6) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10
Equilibrate	Aspirates Equilibration Buffer (deck location 9) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates samples (deck location 5) into the syringes, and then dispenses them through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 8).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–250	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through in the Flow Through Collection plate (deck location 8). If not selected, discards the sample flow-through in the Organic Waste plate (deck location 3).	–	–	–	–
Cup Wash	Rinses the cartridge cups with Equilibration Buffer (deck location 9), and then discards the liquid into the Organic Waste plate (deck location 3).	5 µL:	25	–	3
		25 µL:**	–	–	–
		Range:	0–100	–	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Internal Cartridge Wash	Aspirates Equilibration Buffer (deck location 9) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 8).	5 µL:	50	10	3
		25 µL:**	–	–	–
		Range:	0–250	0.5–500	0–10
Collect Flow Through	Collects the Internal Cartridge Wash flow-through at Flow Through Collection (deck location 8). If not selected, discards the Internal Cartridge Wash flow-through to Organic Waste (deck location 3).	–	–	–	–
Predispense Elution Buffer	Dispenses the specified volume of each Elution Buffer into its respective Fraction Collection plate before the start of each elution.	5 µL:	15	–	–
		25 µL:	15	–	–
		Range:	0–50	–	–
Elute Fraction 1	Aspirates Elution Buffer 1 (deck location 5 following its movement from deck location 4) into the syringes, and then dispenses it through the cartridges into the Fraction Collection Plate 1 (deck location 8 following its movement from deck location 7).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0-250	0.1-500	0–10
Elute Fraction 2	Aspirates Elution Buffer 2 (deck location 5 following its movement from deck location 4) into the syringes, and then dispenses it through the cartridges into the Fraction Collection Plate 2 (deck location 8 following its movement from deck location 7).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0-250	0.1-500	0–10
Elute Fraction 3	Aspirates Elution Buffer 3 (deck location 5 following its movement from deck location 4) into the syringes, and then dispenses it through the cartridges into the Fraction Collection Plate 3 (deck location 8 following its movement from deck location 7).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0-250	0.1-500	0–10
Elute Fraction 4	Aspirates Elution Buffer 4 (deck location 5 following its movement from deck location 4) into the syringes, and then dispenses it through the cartridges into the Fraction Collection Plate 4 (deck location 8 following its movement from deck location 7).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0-250	0.1-500	0–10
Elute Fraction 5	Aspirates Elution Buffer 5 (deck location 5 following its movement from deck location 4) into the syringes, and then dispenses it through the cartridges into the Fraction Collection Plate 5 (deck location 8 following its movement from deck location 7).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0-250	0.1-500	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Elute Fraction 6	Aspirates Elution Buffer 6 (deck location 4) into the syringes, and then dispenses it through the cartridges into the Fraction Collection Plate 6 (deck location 7).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0-250	0.1-500	0–10
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 145.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 154.

**The Cup Wash and Internal Cartridge Wash steps are not an option for the 25 µL cartridges because of the volume limit of the wells in the labware at deck location 9. Instead, a cartridge wash should be done in place of one of the elution steps for the 25 µL cartridges.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT

The protocol will display an error message if cartridges are missing.

Starting the protocol run

WARNING

The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.

To start the Plate Stacking protocol:

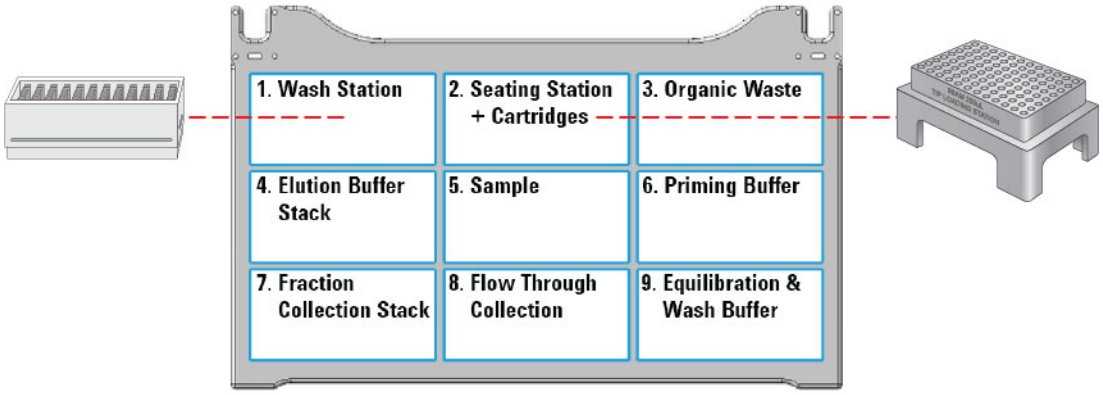
1. Click 

. The Plate Stacking protocol starts.
2. Follow the on-screen instructions to place the Elution Buffer plates at their specified locations. The protocol stacks the Elution Buffer plates at deck location 4.
3. Follow the on-screen instructions to place the Fraction Collection plates at their specified locations. The protocol stacks the Fraction Collection plates at deck location 7.

To start the Fractionation protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

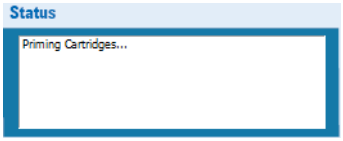
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol. The default protocol with 100-µL samples and 5-µL/min flow rate should take approximately 55 minutes to complete.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

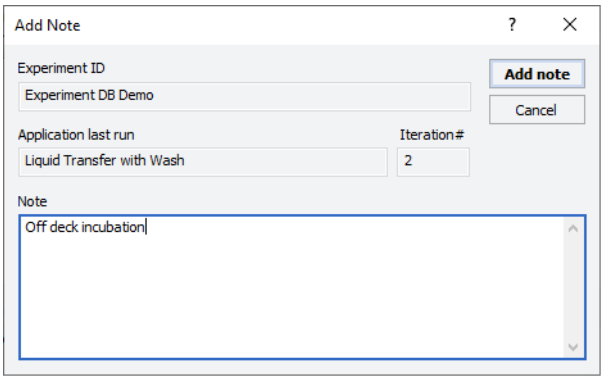
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

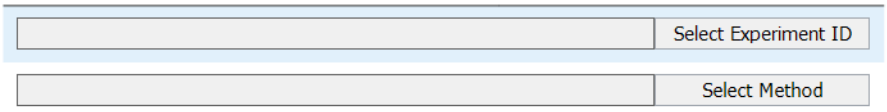
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click Run Protocol to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Fractionation protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines or notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12

Protocol step	Guidelines or notes
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines or notes
Prime	This step removes entrained air from the packed resin bed and properly wets the surface of the resin. In preparation for priming, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The default volume is sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default value diminishes the ability to effectively remove entrained air from the resin bed. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–300 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines or notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP reversed-phase cartridges (C18 and RP-S), which are often used with the Fractionation application, require an equilibration solution that has a very low concentration or no organic solvent for effective binding during the sample loading step. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines or notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the sample loading begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates sample into the syringes and then mounts the cartridges. The samples are dispensed through the cartridges into the Flow Through Collection plate or Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any sample on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. **IMPORTANT** Be sure to include the recommended labware-specific volume overage to prevent air from entering the cartridge. For details, see "Preparing the sample plates" on page 134. This step is selected by default. **Volume (µL).** The volume of sample should be balanced with the sample concentration and the mass capacity of the cartridge. • Default: 100 • Practical: 10–250 • Range: 0–250 *Note:* The lower the sample volume, the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min)**. The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than the default may not significantly increase analyte binding, and using flow rates faster than the default will decrease the quantitative binding capacity of the cartridges. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines or notes
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed into the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step is dispensed into the Organic Waste plate. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is selected by default.
Cup Wash	This step removes the residual sample solution that may remain above the resin bed after the Load Samples step. The Cup Wash step aspirates Wash Buffer into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the Organic Waste plate, and then washing the syringes at the wash station. This step is selected by default for the 5 µL cartridges. The Cup Wash and Internal Cartridge Wash steps are not an option for the 25 µL cartridges because of the volume limit of the wells in the labware at deck location 9. Instead, a cartridge wash should be done in place of one of the elution steps for the 25 µL cartridges. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines or notes
Internal Cartridge Wash	This step uses Wash Buffer to wash non-specifically bound molecules from the resin bed. In preparation for the Internal Cartridge Wash, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Wash Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Wash Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. If the Load Samples step is selected, the first 5 µL (5 µL cartridges) of Wash Buffer is dispensed as a sample chase at the Load Samples flow rate. Next, the Internal Cartridge Wash volume minus the chase volume is dispensed at the Internal Cartridge Wash flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. This step is selected by default for the 5 µL cartridges. The Cup Wash and Internal Cartridge Wash steps are not an option for the 25 µL cartridges because of the volume limit of the wells in the labware at deck location 9. Instead, a cartridge wash should be done in place of one of the elution steps for the 25 µL cartridges. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except for syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min might not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines or notes
Collect Flow Through	If this step is selected, the flow-through from Internal Cartridge Wash step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from Internal Cartridge Wash is dispensed into the Organic Waste plate. This step is selected by default. This step is skipped if the Internal Cartridge Wash step is not conducted.
Predispense Elution Buffer	This step maximizes the recovery of small volume elutions (< ~ 20 µL) by allowing direct elution into a liquid. Small volumes may cling to the end of the cartridge during elution that cannot be removed by the programmed tip touches in the wells of the plates. This step transfers a specified volume of Elution Buffer from the appropriate Elution Buffer plate (1-6) to the corresponding Fraction Collection plate before eluting each fraction into the Fraction Collection plate. **Volume (µL):** • Default: 15 • Range: 0–50
Elute Fraction 1–6	Before running the Fractionation protocol, the Stack Plates protocol stacks a set of Elution Buffer plates at deck location 4 and a set of Fraction Collection plates at deck location 7. The number of Fraction Collections plates is equal to the Number of Fractions you specified. In preparation for elution, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Elution Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During this step, the plate movements on the deck are as follows: **1** The Sample plate and Flow Through Collection plate move one position to the right and are stacked on the Priming Buffer plate and Equilibration Buffer plate, respectively. **2** To collect fractions 1-4, the Elution Buffer and Fraction Collection plates are moved from the stacks a locations 4 and 7 to deck locations 5 and 8 before each elution step. **3** The Elution Buffer and Fraction Collection plates for fractions 1–4 are stacked at deck locations 6 and 9 after each elution step. For example: Elution Buffer plate 1 is moved from the Elution Buffer plates stacked at location 4 to location 5. Fraction Collection plate 1 is moved from the Fraction Collection plates stacked at location 7 to location 8. Elution buffer is aspirated into the syringes from Elution Buffer Plate 1 at location 5. If the Predispense Elution Buffer step was selected, this volume will be dispensed to Fraction Collection plate 1 at location 8. Cartridges are mounted and samples are eluted from the cartridge directly into Fraction Collection plate 1. The cartridges are parked and the syringes are washed at the wash station. Fraction Collection plate 1 is moved from location 8 and stacked on location 9. Elution Buffer plate 1 is moved from location 5 and stacked on location 6.

Protocol step	Guidelines or notes
	4 For fraction 5, step 2 is conducted but instead of stacking the Elution Buffer and Fraction Collection plates (step 3), they remain at locations 5 and 8, respectively. **5** For fraction 6, there is no plate movement. Elution Buffer plate 6 remains at location 4 and Fraction Collection plate 6 remains at location 7. Final Fraction Collection plates 1-4 will be in deck location 9, Fraction Collection plate 5 will be in deck location 8 and Fraction Collection plate 6 will be in deck location 7. **Volume (µL)**. The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges, or 50–75 µL for 25 µL cartridges). The default volumes are conservative and significantly higher than the minimum expected with a strong Elution Buffer. • Volume for 5 µL cartridges: – Default: 25 – Practical: 10–30 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 125 – Practical: 50–150 – Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing. **Flow rate (µL/min).** A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more elution buffer may be required to get the same elution yield at high elution flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500 **Wash cycle**. The number of syringe washes to perform at the wash station after an Elute step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines or notes
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, the 10 µL of elution buffer will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

Stack Plates protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Stack Plates protocol. Changing the Number of Fractions will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	4	Checks the labware height.
	7	Checks the labware height.
Stack Elution Buffer plates	8 to 4	Moves the Elution Buffer plate from deck location 8 to 4.
	5 to 4 9 to 4 6 to 4 3 to 4	Moves the remaining Elution Buffer plates, in the order listed, to deck location 4.
Stack Fraction Collection plates	8 to 7	Moves the Fraction Collection plate from deck location 8 to 7.
	5 to 7 9 to 7 6 to 7 3 to 7	Moves the remaining Fraction Collection plates, in the order listed, to deck location 7.

Fractionation protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Fractionation protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	4	Checks the Elution Buffer plate stack height.
	7	Checks the Fraction Collection plate stack height.
	9	Checks the labware height.
	6	Checks the labware height.
	2	Parks all cartridges that might have been loaded on the head from a previously aborted protocol.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes.
Prime Cartridges	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Performs an external probe wash.
	6	Aspirates 10 µL of Priming Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates the Priming Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Priming Buffer through the cartridges and into Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Equilibrate Cartridges	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Performs an external probe wash.
	9	Aspirates 10 µL of Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Equilibration Buffer into the cartridge cups and exercises the cartridges off task.
	9	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Equilibration Buffer through the cartridges into the Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Load Samples	5	Aspirates the samples into the syringes.
	2	Mounts the cartridges on the head.
	8	Dispenses the samples through the cartridges and into the Flow through Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash (5 µL cartridges only)	9	Aspirates the Wash Buffer.
	2	Washes the cartridge cups and exercises the cartridges off task.
	3	Dispenses the Wash Buffer into the Organic Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash (5 µL cartridges only)	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Performs an external probe wash.
	9	Aspirates 10 µL of Wash Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Wash Buffer into the cartridge cups and exercises the cartridges off task.
	9	Aspirates the Wash Buffer.
	2	Mounts cartridges on the head.
	3	Dispenses 5 µL Wash Buffer through the cartridges at the Load Samples flow rate for the sample chase step.
	3	Dispenses the remaining Wash buffer through the cartridges at the Internal Cartridge Wash flow rate and into the Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
	5	Moves the Sample plate to deck location 6.
	8	Moves the Flow Through Collection plate to deck location 9.

Protocol step	Head moves to deck location...	Action
Elute Fractions 1–6	4 to 5	Moves Elution Buffer plate from deck location 4 to 5.
	7 to 8	Moves Fraction Collection plate from deck location 7 to 8.
	5	Aspirates the Elution Buffer for elution.
	2	Mounts the cartridges on the head.
	8	Elutes into the Fraction Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
	8 to 9	Moves Fraction Collection plate from deck location 8 to 9.
	5 to 6	Moves Elution Buffer plate from deck location 5 to 6.
	Repeats for fractions 2, 3, and 4.	
	Repeats for fraction 5, except the final plates remain at deck locations 5 and 8.	
	Repeats for fraction 6, except the plates remain at deck locations 4 and 7. *Note:* Final Fraction Collection plates 1-4 will be at deck location 9. Fraction Collection plate 5 will be in deck location 8. Fraction Collection plate 6 will be at deck location 7.	
Final Syringe Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the syringes.

Reference library

1 Russel, J., Van Den Heuvel, Z., Bovee, M. & Murphy, S., Workflow Automation for LC/MS: In-Solution Protein Digestion, Peptide Cleanup, and Strong Cation-Exchange Fractionation of Peptides Enabled by AssayMAP Technology, Agilent Application Note 5991-3602EN, 2013

2 Russel, J., Van Den Heuvel, Z., Bovee, M., Murphy, S., Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-2957EN, 2016

See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Bravo Platform.

6 IMAC Cartridge Customization v2.0 User Guide

This chapter contains the following topics:

App description

IMAC Cartridge Customization v2.0. This application enables automated stripping and charging of AssayMAP Fe(III)-NTA cartridges with the desired metal of choice for immobilized metal affinity chromatography (IMAC) experiments. The protocol enables metal customization of from 1 to 96 cartridges in a single run.

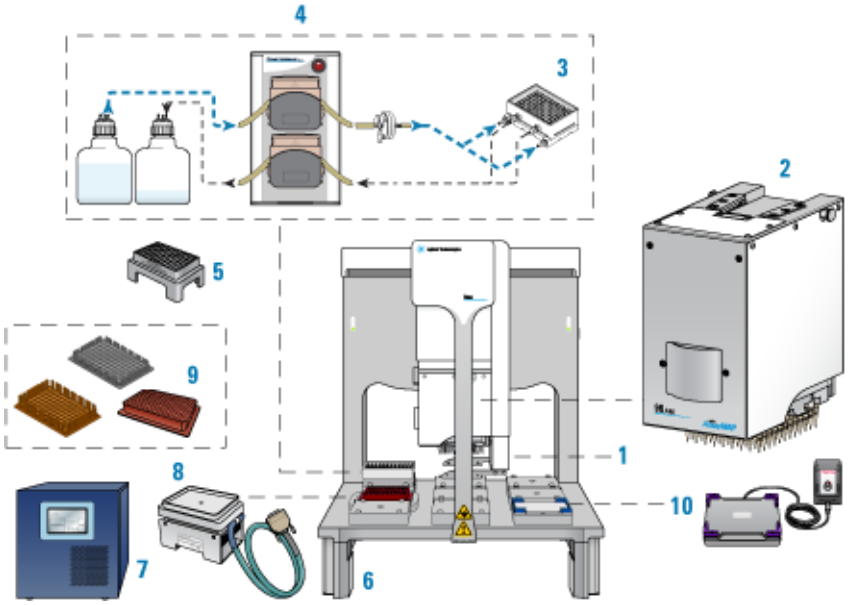
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the IMAC Cartridge Customization protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. You might need the following when preparing the reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent

- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

AssayMAP Fe(III)-NTA cartridges are available for performing phosphopeptide enrichment using the AssayMAP Bravo Platform. While Fe(III) is currently the most popular choice of metal cation for phosphopeptide enrichment, other metal cations such as Ga(III) and Zr(IV) have been proven effective for phosphopeptide enrichment using immobilized metal affinity chromatography (IMAC) (ref 1–6). In addition, AssayMAP Fe(III)-NTA cartridges can be functionalized with metals such as Ni(II), Co(II), Zn(II), or Cu(II) to perform IMAC purification of histidine-tagged proteins or metal-binding proteins.

The following table lists the available AssayMAP cartridges for performing IMAC Cartridge Customization on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Fe(III)-NTA cartridge rack	G5496-60085	–
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or to fill remaining positions in columns that are partially occupied by Fe(III)-NTA cartridges, if necessary. For details, see "Preparing the buffers and reagents" on page 167.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.
- AssayMAP Fe(III)-NTA cartridges are stable from approximately pH 2 to 11. At levels approximately > pH 3.5, resin within the cartridges may turn from pale yellow to golden yellow to orange or brown due to the formation of iron(III) complexes (most commonly with hydroxide). Resin coloration and intensity are a function of both the pH and the chemical nature of the solutions passed through the cartridge. Bare NTA cartridges are stable from approximately pH 2 to 14.
- AssayMAP Fe(III)-NTA cartridges that have been stripped (bare NTA cartridges) should be charged and used immediately.

Labware

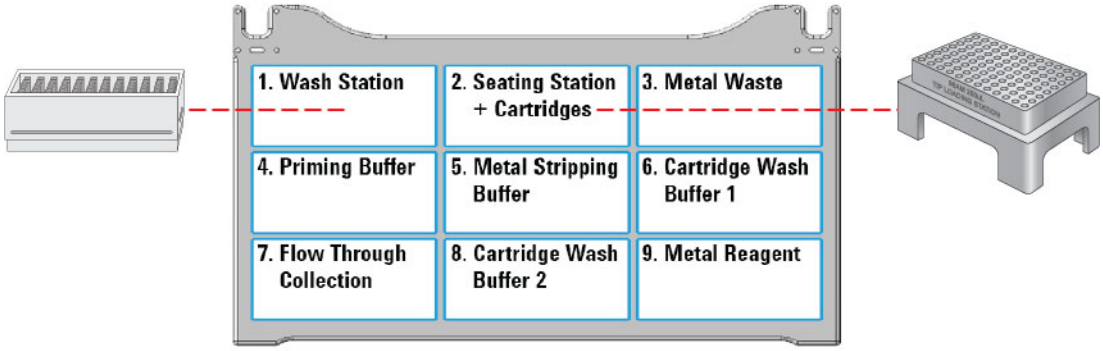
Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware	Mfr part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3—9
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3—9
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—8
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	7, 9**
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	7, 9**
96 Greiner 650201 U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3—9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	4, 5, 6, 8, 9
Reservoir, Axygen Scientific RES-SW96-LP, 86mL, pyramid bottom	Axygen Scientific RES-SW96-LP	4, 5, 6, 8, 9
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottoms	Agilent 201254-100	4, 5, 6, 8, 9

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner and BioRad PCR plates are not compatible with the 25 µL cartridges at deck location 7.

Reagents

The volume, type, and concentration of reagents required for cartridge customization vary depending on sample characteristics and the desired analytical result. Consult published literature ("Reference library" on page 192) for reagent recommendations for

sample and surface chemistry combinations. Also, see the Agilent AssayMAP Bravo Citation Index for published papers that use the IMAC Cartridge Customization application on the AssayMAP Bravo Platform.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 165.

Preparing the solutions

The following solutions are required for the IMAC Cartridge Customization protocol:

- Priming Buffer
- Metal Stripping Buffer
- Cartridge Wash Buffers
- Metal Reagent

CAUTION

A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Metal-binding capacity

The metal-binding capacity of each AssayMAP NTA cartridge is >100 nmol of Fe(III) per cartridge as determined by inductively coupled plasma—optical emission spectroscopy (ICP-OES).

Using the Reagent Volume Calculator for IMAC Cartridge Customization

The Reagent Volume Calculator is a Microsoft Excel file that contains a Calculator worksheet. You enter the number of columns to process, whether to perform the Collect Flow Through options, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

To use the Reagent Volume Calculator:

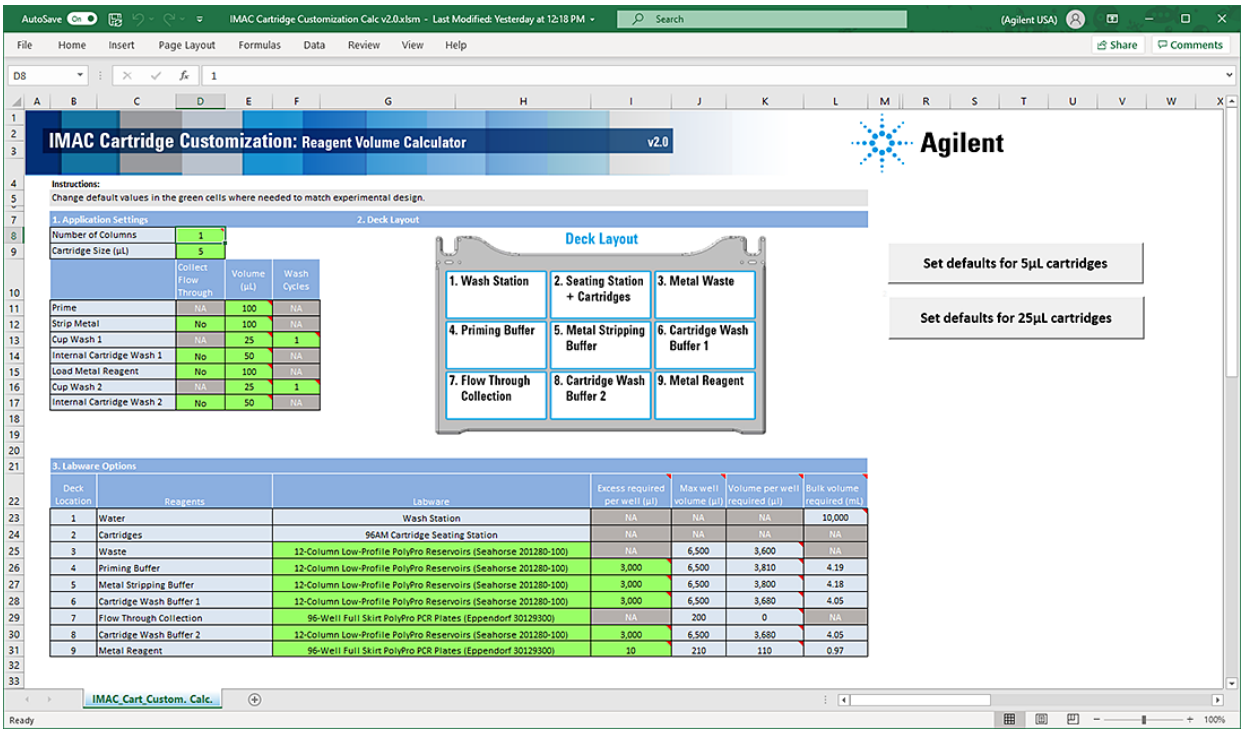
1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figure shows the Reagent Volume Calculator.

Figure IMAC Cartridge Customization Calculator worksheet



Removing macromolecular particulates

Make sure the solutions are free of macromolecular particulates, such as undissolved or precipitated salts. Use a 0.22-µm filter to filter any salt-containing solutions and minimize the possibility of clogging the AssayMAP cartridges.

CAUTION

A build-up of salts within the syringe barrels can corrode the syringe seals. Therefore, you should filter salt-containing buffers before use. In addition, you should use the "System Startup/Shutdown v3.0 User Guide" on page 574 **utility to clean the syringes after every protocol run.**

Preparing the buffers and reagents

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the protocol for stripping and charging Fe(III)-NTA cartridges.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming Buffer (deck location 4)	To properly wet the surface of the resin and remove entrained air, use a priming solution containing a high amount of organic solvent. Organic solvent in the solution helps to purge entrained air within the cartridge resin bed and ensure the resin is properly wetted. The following buffer has been used successfully with the Fe(III)-NTA cartridges. 50% ACN : 50% H_2O
Metal Stripping Buffer (deck location 5)	A chelator is necessary to remove the pre-charged iron from AssayMAP Fe(III)-NTA cartridges. Ethylenediaminetetraacetic acid is a strong chelator of metal cations and an aqueous solution is able to strip away metals bound to the NTA resin at a pH that is neutral to slightly basic. The following buffer has been used successfully to strip metal from the Fe(III)-NTA cartridges. 100 mM EDTA, pH 8.0
Cartridge Wash Buffer 1 (deck location 6)	This aqueous solution is used to flush away any remaining Metal Stripping Buffer and prepares the cartridge resin with a solution compatible with the Metal Reagent. The following buffer has been used successfully with the Fe(III)-NTA cartridges. 10 mM HCl, 50 mM acetic acid, or 0.1% TFA

Reagent (deck location)	Composition and comments
Metal Reagent (deck location 9)	This aqueous solution is used to immobilize metal cations to the surface of the bare NTA cartridge. Many aqueous metal solutions are highly acidic (pH < 1). Brief exposure of the resin bed to low-pH conditions during the Load Metal step will not harm the NTA resin within cartridges. The following reagent has been used successfully with the Fe(III)-NTA cartridges. 50–150 mM metal salt in H_2O
Cartridge Wash Buffer 2 (deck location 8)	This aqueous solution flushes any remaining Metal Reagent from the cartridges and leaves them in a state ready for immediate use or for short-term storage. For storage guidelines, see "AssayMAP cartridges" on page 162. Cartridge Wash Buffer 2 should not cause metal precipitation, form highly stable metal complexes with the immobilized metal, or contain a strong chelator that could strip the immobilized metal from cartridges. Consult relevant literature for specific metals to determine chemical compatibility of selected reagents. The following buffer has been used successfully to load Fe(III) on the Fe(III)-NTA cartridges that have been stripped of Fe(III) using this application. 10 mM HCl, 50 mM acetic acid, or 0.1% TFA

Note: All suggested solutions listed as percentages are volume/volume formulations.

Dispensing the solutions

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes and subsequently be dispensed into the resin bed, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well. See "Using the Reagent Volume Calculator for IMAC Cartridge Customization" on page 165.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the cartridge positions in the 96AM Cartridge & Tip Seating Station. See "Planning the cartridge layout" on page 169.

To dispense the solutions into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Priming Buffer into the plate or reservoir to be placed at deck location 4.
3. Add the specified volume of Metal Stripping Buffer into the plate or reservoir to be placed at deck location 5.
4. Add the specified volume of Cartridge Wash Buffer 1 into the plate or reservoir to be placed at deck location 6.
5. Add the specified volume of Cartridge Wash Buffer 2 into the plate or reservoir to be placed at deck location 8.
6. Add the specified volume of Metal Reagent into the plate or reservoir to be placed at deck location 9.
7. If necessary, centrifuge the buffer and reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

See "Labware" on page 163 for acceptable labware at each deck location.

Planning the cartridge layout

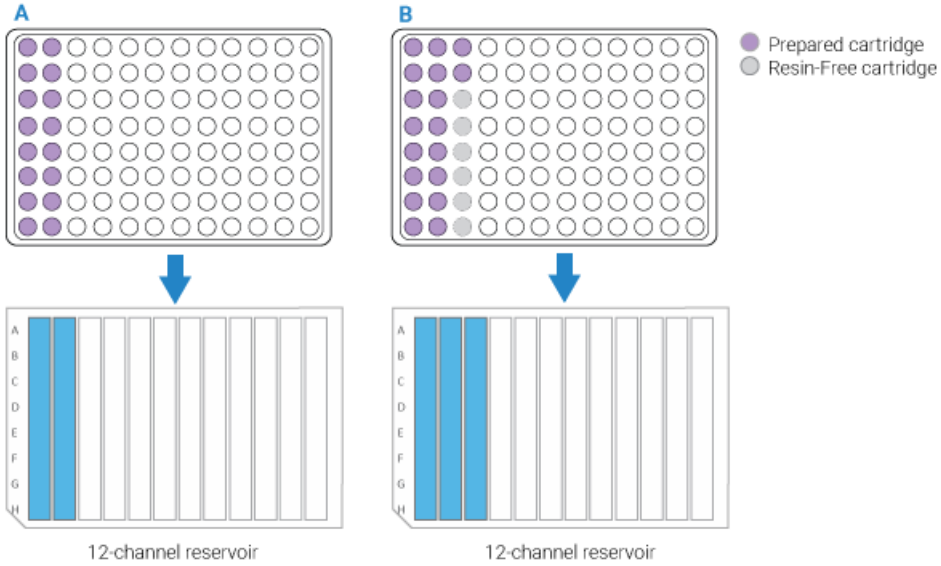
Before transferring the reagents, you should plan the layout of the cartridges in the microplate. Consider the following:

- You can process 1 to 96 AssayMAP Fe(III)-NTA cartridges in parallel. The position of the cartridges in the seating station dictates the position of the reagent and buffer solutions in the microplates and reservoirs.
- If you have fewer than 96 Fe(III)-NTA cartridges, make sure the cartridges occupy full columns in the seating station, as the figure below shows.

  The default protocol settings assume that cartridges will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based on the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.

- If the number of Fe(III)-NTA cartridges you have is not a multiple of 8, use AssayMAP Resin-Free cartridges to fill the empty well positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash steps.

Figure Examples of cartridge and reservoir layout: A) Multiple of 8 Fe(III)-NTA cartridges, B) Fe(III)-NTA cartridges and Resin-Free cartridges



Running the protocol

The IMAC Cartridge Customization protocol:

- Washes the syringes.
- Primes the cartridges.
- Strips the iron from the cartridges.
- Removes the Metal Striping Buffer from the cartridges.
- Charges the cartridges with the desired metal of choice.
- Removes unbound metal from the cartridges.

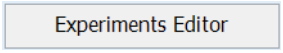
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the cartridge seating station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the buffers and reagents. See "Preparing the solutions" on page 165.

- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the 96AM Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

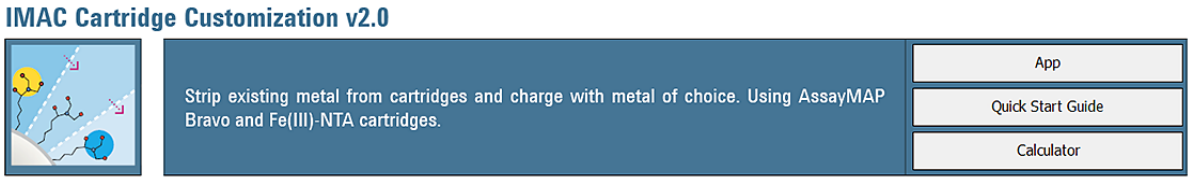
IMPORTANT Do not allow wetted cartridges to dry out. See "Cartridge use and storage guidelines" on page 163.

Setting up the protocol

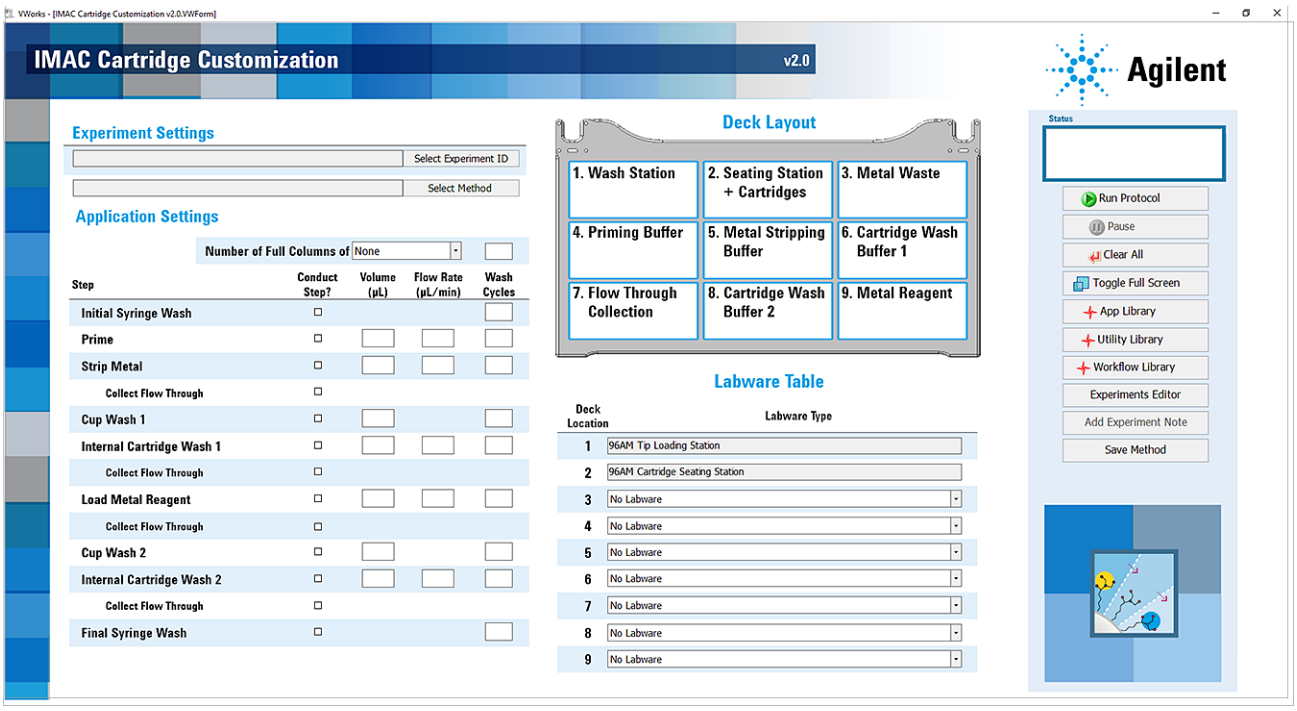
Before starting the protocol, make sure the appropriate selections and values are specified in the IMAC Cartridge Customization application.

To set up the protocol:

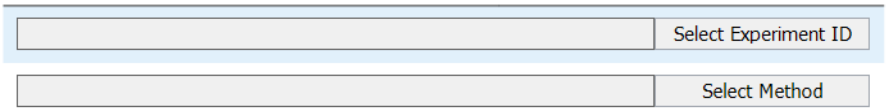
1. Open the **App** library.
2. Locate **IMAC Cartridge Customization**, and then click **App**.



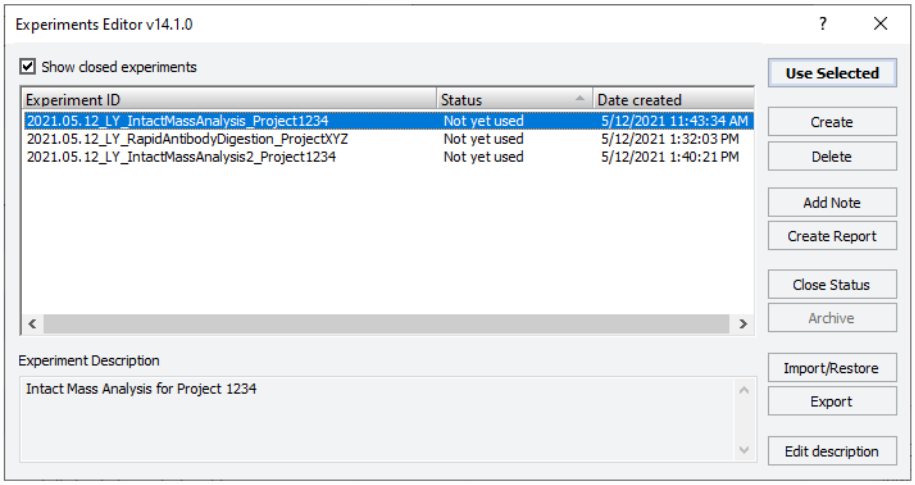
The IMAC Cartridge Customization application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

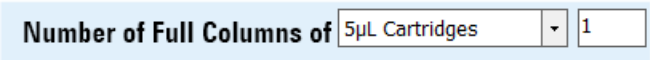
5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 177.
- To modify the method or create a method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:



a Select the cartridge size from the list:
- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION **If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.**

CAUTION **If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.**

IMPORTANT Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 179.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 4) into the syringes, and then dispenses it through the cartridges into the Waste plate (deck location 3).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10
Strip Metal	Aspirates Metal Stripping Buffer (deck location 5) into the syringes, and then dispenses it through the cartridges into Flow Through Collection (deck location 7), or into the Waste plate (deck location 3).	5 µL:	50	5	3
		25 µL:	250	5	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Strip Metal flow-through at Flow Through Collection (deck location 7). If not selected, discards the flow-through into the Waste plate (deck location 3).	–	–	–	–
Cup Wash 1	Rinses the cartridge cups with Cartridge Wash Buffer 1 (deck location 6), and then discards the liquid into the Waste plate (deck location 3).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 1	Aspirates Cartridge Wash Buffer 1 (deck location 6) into the syringes, and then dispenses it through the cartridges into Flow Through Collection (deck location 7), or into the Waste plate (deck location 3).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Collect Flow Through	If selected, collects the Internal Cartridge Wash 1 flow-through at Flow Through Collection (deck location 7). If not selected, discards the Internal Cartridge Wash 1 flow-through into the Waste plate (deck location 3).	–	–	–	–
Load Metal Reagent	Aspirates Metal Reagent (deck location 9) into the syringes, and then dispenses through the cartridges into Flow Through Collection (deck location 7), or into the Waste plate (deck location 3).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–250	0.1–500	0–10
Collect Flow Through	If selected, collects the flow-through from Load Metal Reagent at Flow Through Collection (deck location 7). If not selected, discards the Load Metal Reagent flow-through into the Waste plate (deck location 3).	–	–	–	–
Cup Wash 2	Rinses the cartridge cups with Cartridge Wash Buffer 2 (deck location 8), and then discards the liquid into the Waste plate (deck location 3).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 2	Aspirates Cartridge Wash Buffer 2 (deck location 8) into the syringes, and then dispenses it through the cartridges into Flow Through Collection (deck location 7), or into the Waste plate (deck location 3).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash 2 flow-through at Flow Through Collection (deck location 7). If not selected, discards the Internal Cartridge Wash 2 flow-through into the Waste plate (deck location 3).	–	–	–	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see the "Protocol stepwise guidelines" on page 180.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 188.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT The protocol will display an error message if cartridges are missing.

Starting the protocol run

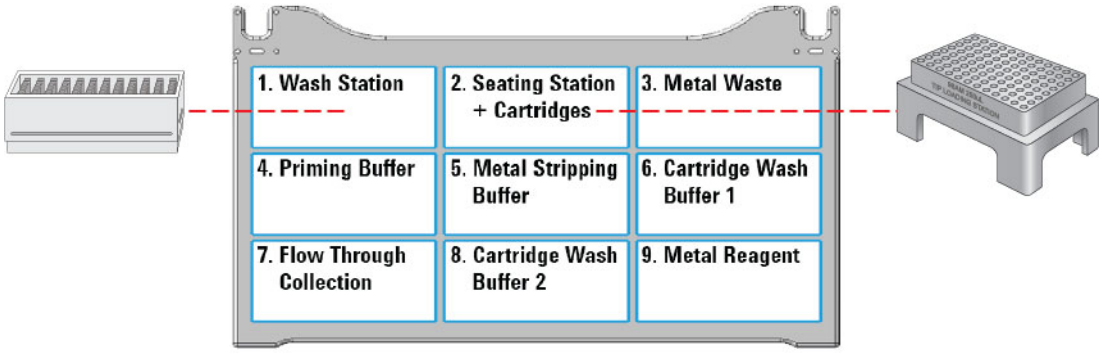
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

Note: The Greiner and BioRad PCR plates are not compatible with the 25 µL cartridges at deck location 7.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

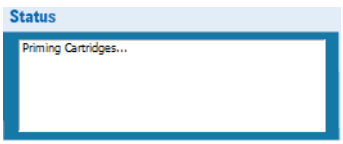
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol. The default protocol should take approximately 60 minutes to complete.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

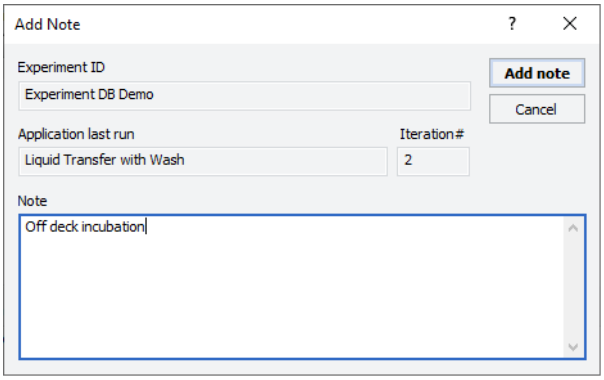
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

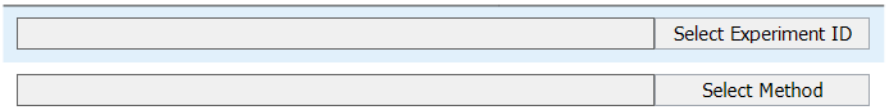
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the IMAC Cartridge Customization protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the resin bed and properly wets the surface of the resin. In preparation for priming, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Metal Waste plate, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Metal Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. For the most effective priming of AssayMAP Fe(III)-NTA cartridges, the Priming Buffer requires that the solution contain at least 50% organic solvent. Higher concentrations of organic solvent are also acceptable. This step is selected by default. **Volume (µL)**. The default volume is sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default value diminishes the ability to effectively remove entrained air from the cartridge. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Strip Metal	This step removes the metal coordinated to the Fe(III)-NTA resin by using a strong chelator, such as EDTA. Complete removal of the bound metal from the resin bed is critical so that a different metal can be bound at the maximum metal-binding capacity of the cartridge. In preparation for stripping, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Metal Waste plate, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Strip Metal step aspirates the specified volume of Metal Stripping Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through cartridges into the Metal Waste plate or into the Flow Through Collection plate. The exterior of the cartridge tips are washed at the wash station to remove any residual buffer on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete removal of bound metal using a sufficiently concentrated chelator in the Metal Stripping Buffer. The default volume is probably much higher than required to strip the metal from the cartridges, but lower volumes have not been tested. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Strip Metal step tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default will likely have no benefit, but will increase the total assay time. A flow rate > 15 µL/min using the default volume might not permit full penetration into the pores of the resin across the full length of the cartridge bed leaving residual metal on the cartridge. • Default: 5 • Practical: 2–15 • Range: 0.5–500 **Wash cycle**.The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the flow-through from the Strip Metal step is dispensed directly into the Flow Through Collection plate. If this step is not selected, the flow-through is dispensed directly into the Metal Waste plate. This step is not selected by default.
Cup Wash 1	This step removes the small volume of residual liquid that might remain above the resin bed after the Strip Metal step. The Cup Wash step aspirates Cartridge Wash Buffer 1 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the Metal Waste plate, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 1	This step uses Cartridge Wash Buffer 1 to wash any remaining Metal Stripping Buffer from the resin bed and equilibrate the resin bed with a buffer compatible with the Metal Reagent to be used in the Load Metal step. In preparation for Internal Cartridge Wash 1, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Metal Waste plate, 10 µL of Cartridge Wash Buffer 1 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 1 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or the Metal Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the flow-through from the Internal Cartridge Wash 1 step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from Internal Cartridge Wash 1 is dispensed into the Metal Waste plate. This step is not selected by default. For the maximum practical working volumes per well for specific labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.
Load Metal Reagent	This step allows the target metal to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the metal loading begins. The assumption is that there is still liquid in the cups from the wash step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates Metal Reagent into the syringes, and then performs an external syringe wash at the wash station to remove any Metal Reagent remaining on the outside of the probes before mounting the cartridges. The Metal Reagent is dispensed through the cartridges into the Flow Through Collection plate or the Metal Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any Metal Reagent on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The volume of the Metal Reagent should be balanced with the concentration of the Metal Reagent and the metal binding capacity of the cartridge to ensure a large molar excess of metal to metal binding sites in the resin bed. • Default: 100 • Practical: 50–150 • Range: 0–250 *Note:* Setting the volume to zero skips all Load Metal Reagent tasks except syringe washing. **Flow rate (µL/min)**. A flow rate less than the default will likely have no benefit, but will increase the total assay time. A flow rate > 15 µL/min using the default volume might not permit full penetration into the pores of the resin across the full length of the resin bed, leaving portions of uncharged NTA in the resin bed, which would reduce binding capacity. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the flow-through from the Load Metal Reagent step is dispensed into the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Metal Reagent step is dispensed into the Metal Waste plate. This step is not selected by default.
Cup Wash 2	This step removes the residual solution that may remain above the resin bed after the Load Metal Reagent step. The Cup Wash 2 step aspirates Cartridge Wash Buffer 2 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the Metal Waste plate, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. A volume less than the default might be insufficient for cup washing, while a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 2	This step uses Cartridge Wash Buffer 2 to wash unbound metal from the resin bed. In preparation for Internal Cartridge Wash 2, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Metal Waste plate, 10 µL of Cartridge Wash Buffer 2 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 2 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or the Metal Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to 0 skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min might not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the flow-through from the Internal Cartridge Wash 2 step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from the Internal Cartridge Wash 2 step is dispensed directly into the Metal Waste plate. This step is not selected by default.

Protocol step	Guidelines and notes
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the Final Syringe Wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Metal Waste plate. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, 10 µL of Cartridge Wash 2 will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL of DI water into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. In cases where carryover is a major concern, increasing the number of wash cycles may provide marginally improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic automation movements of the AssayMAP Bravo Platform during the protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	2	Parks all cartridges that might have been loaded on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes for 3 cycles.

Protocol step	Head moves to deck location...	Action
Prime	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Metal Waste plate.
	1	Washes the exterior of the syringe probes.
	4	Aspirates 10 µL of Priming Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	4	Aspirates the Priming Buffer.
	2	Mounts the cartridges onto the head.
	3	Dispenses the Priming Buffer through the cartridges into the Metal Waste plate to prime the cartridges.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Strip Metal	5	Aspirates the Metal Stripping Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Metal Stripping Buffer through the cartridges into the Metal Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash 1	6	Aspirates the Cartridge Wash Buffer 1 into the syringes.
	2	Washes the cartridge cups and exercises the cartridges off task.
	3	Dispenses the Cartridge Wash buffer 1 into the Metal Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash 1	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Metal Waste plate.
	1	Washes the exterior of the syringe probes.
	6	Aspirates 10 µL of Cartridge Wash Buffer 1 for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates the Cartridge Wash Buffer 1 into the syringes.
	2	Mounts the cartridges on the head.
	3	Dispenses the Cartridge Wash Buffer 1 through the cartridges into the Metal Waste plate.
	1	Washes the exterior of the cartridge tips at the wash station.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Load Metal Reagent	9	Aspirates the Metal Reagent into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	3	Dispenses the Metal Reagent through the cartridges into the Metal Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash 2	8	Aspirates the Cartridge Wash Buffer 2 into the syringes.
	2	Washes the cartridge cups and exercises the cartridges off task.
	3	Dispenses the Cartridge Wash buffer 2 into the Metal Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash 2	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Metal Waste plate.
	1	Washes the exterior of the syringe probes.
	8	Aspirates 10 µL of Cartridge Wash Buffer 2 for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates Cartridge Wash Buffer 2 into the syringes.
	2	Mounts the cartridges on the head.
	3	Dispenses Cartridge Wash Buffer 2 through the cartridges into the Metal Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Final Syringe Wash	2	Moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Metal Waste plate.
	1	Washes the syringes.

Reference library

1 Posewitz, M.C. & Tempst, P. Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal. Chem.*, 1999, 71:2883-2892.

2 Seeley, E.H., Riggs, L.D. & Regnier, F.E. Reduction of non-specific binding in Ga(III) immobilized metal affinity chromatography for phosphopeptides by using endoproteinase glu-C as the digestive enzyme. *J. Chromatogr. B*, 2005, 817:81-88.

3 Steen, H., Stensballe, A. & Jensen, O.N. Phosphopeptide purification by IMAC with Fe(III) and Ga(III). *Cold Spring Harbor Protocols*, 2007, 2007:pdb.prot4607.

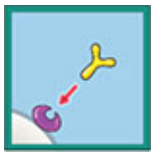
4 Machida, M. et al. Purification of phosphoproteins by immobilized metal affinity chromatography and its application to phosphoproteome analysis. *FEBS J.*, 2007, 274:1576-1587.

5 Feng, S. et al. Immobilized zirconium ion affinity chromatography for specific enrichment of phosphopeptides in phosphoproteome analysis. *Mol. Cell. Proteomics*, 2007, 6:1656-1665.

6 Hsu, J.L. et al. Functional phosphoproteomic profiling of phosphorylation sites in membrane fractions of salt-stressed Arabidopsis thaliana. *Proteome Sci.*, 2009, 7:42.

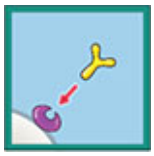
See the Agilent AssayMAP Bravo Citation Index for published papers that used the AssayMAP Bravo Platform.

7 Immobilization v3.0 User Guide



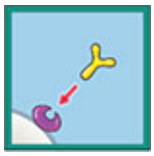
This chapter contains the following topics:

App description



Immobilization v3.0. This application enables the creation of custom affinity purification cartridges by automating the immobilization of antibodies and other affinity ligands, from 1 to 96 standard AssayMAP cartridges in a single run.

Before you start

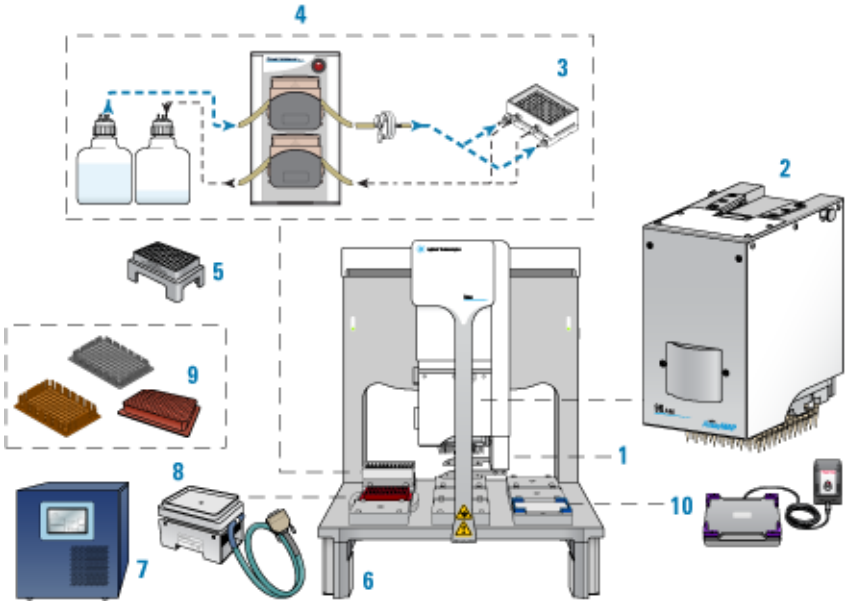


This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Immobilization protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION

To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Immobilization and Affinity Purification protocols on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Protein A (PA-W) cartridge rack	G5496-60000	G5496-60018
AssayMAP Protein G (PG-W) cartridge rack	G5496-60008	–
AssayMAP Streptavidin (SA-W) cartridge rack	G5496-60010	G5496-60021
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of Protein A, Protein G, or Streptavidin 5- or 25-µL cartridges are required. For details, see "Preparing the samples" on page 203.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.
- PA-W, SA-W, and PG-W cartridges tolerate brief exposure to pH as low as 2.0. The stability of the PA-W, SA-W, and PG-W cartridges after capturing additional affinity ligands should be determined empirically.

Labware

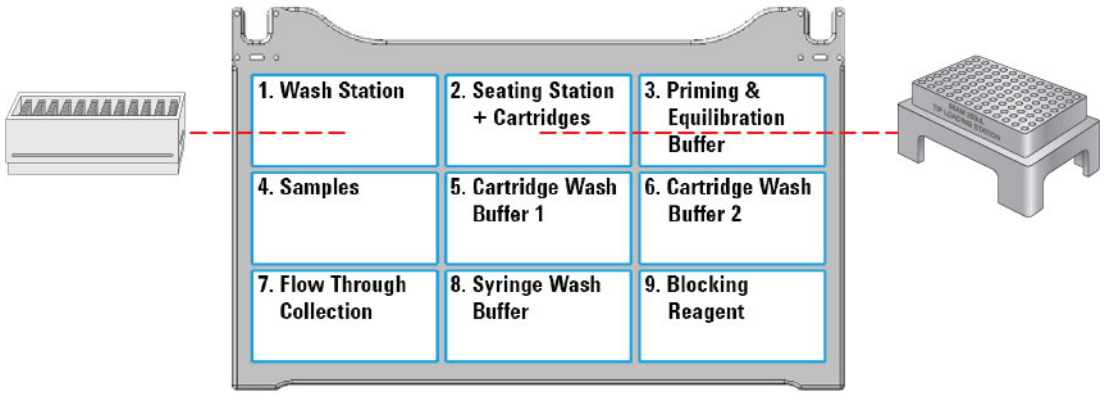
Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware	Mfr part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3, 5, 6, 7, 8

Labware	Mfr part number*	Deck location options
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3, 5, 6, 7, 8
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—8
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7, 9**
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3—9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	4, 7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	3, 5, 6, 8

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner PCR plate is not compatible with the 25 µL cartridges at deck location 7.

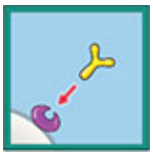
Reagents

The volume, type, and concentration of reagents required to immobilize antibodies or other affinity ligands onto AssayMAP cartridges for subsequent affinity purification experiments will vary depending on sample characteristics and the desired analytical result. Consult the resources that cover general principles of affinity purification such as those listed in the "Reference library" on page 234. For examples of reagents used during the immobilization of affinity ligands to protein A, protein G, and Streptavidin, consult the published scientific literature including the publications that use the AssayMAP Bravo Platform listed in the Agilent AssayMAP Bravo Citation Index.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure due to the buildup of salts within the syringe barrels. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Immobilization Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 199.

Preparing the solutions



The following solutions are used for the Immobilization protocol:

- Priming & Equilibration Buffer
- Wash Buffers: Cartridge Wash 1, Cartridge Wash 2, and Stringent Syringe Wash
- Blocking Reagent

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Immobilization Reagent Volume Calculator

The Reagent Volume Calculator is a Microsoft Excel file that contains the following:

- *Calculator worksheet*. You enter the number of columns to process, whether to perform the Collect Flow Through option, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

  Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.
- *Reagent Recipe worksheet*. You enter the concentrations of each component in your reagent, and the worksheet calculates the recipe volumes required.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figures show the worksheets of the Reagent Volume Calculator.

Figure Immobilization Reagent Volume Calculator worksheet

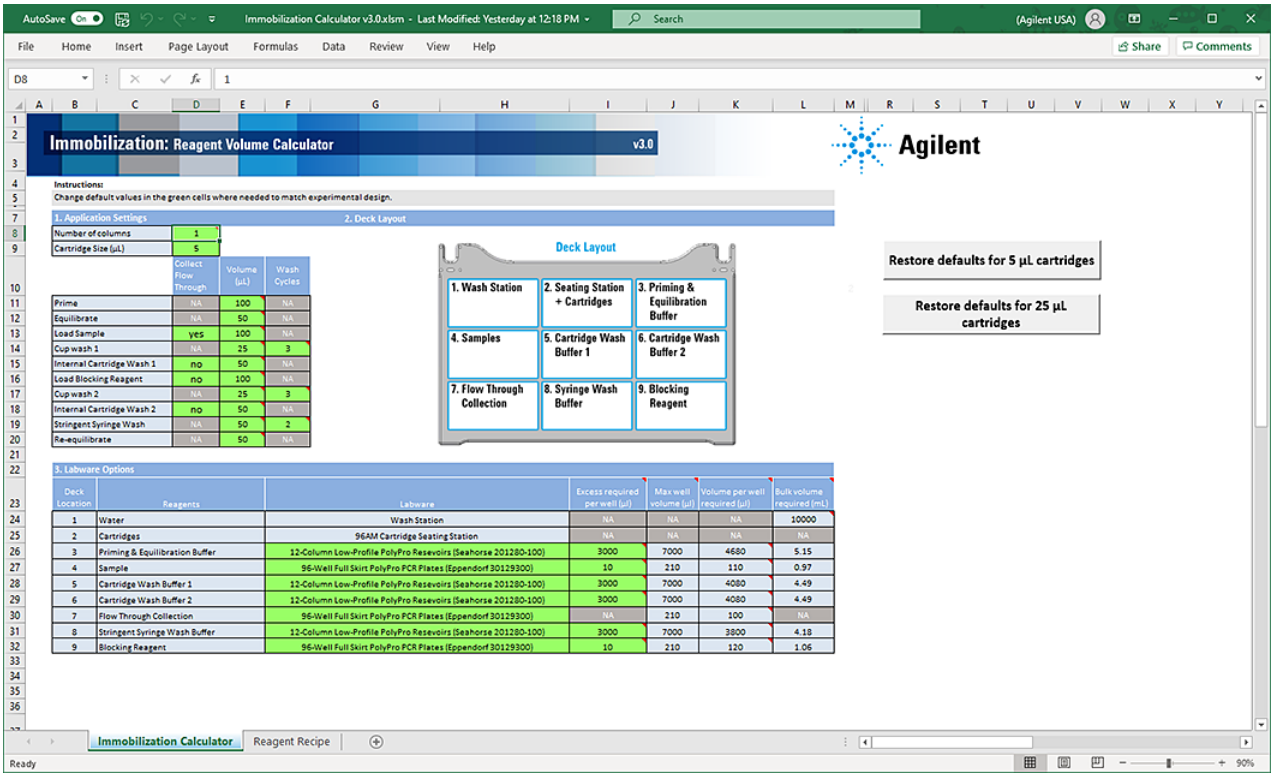
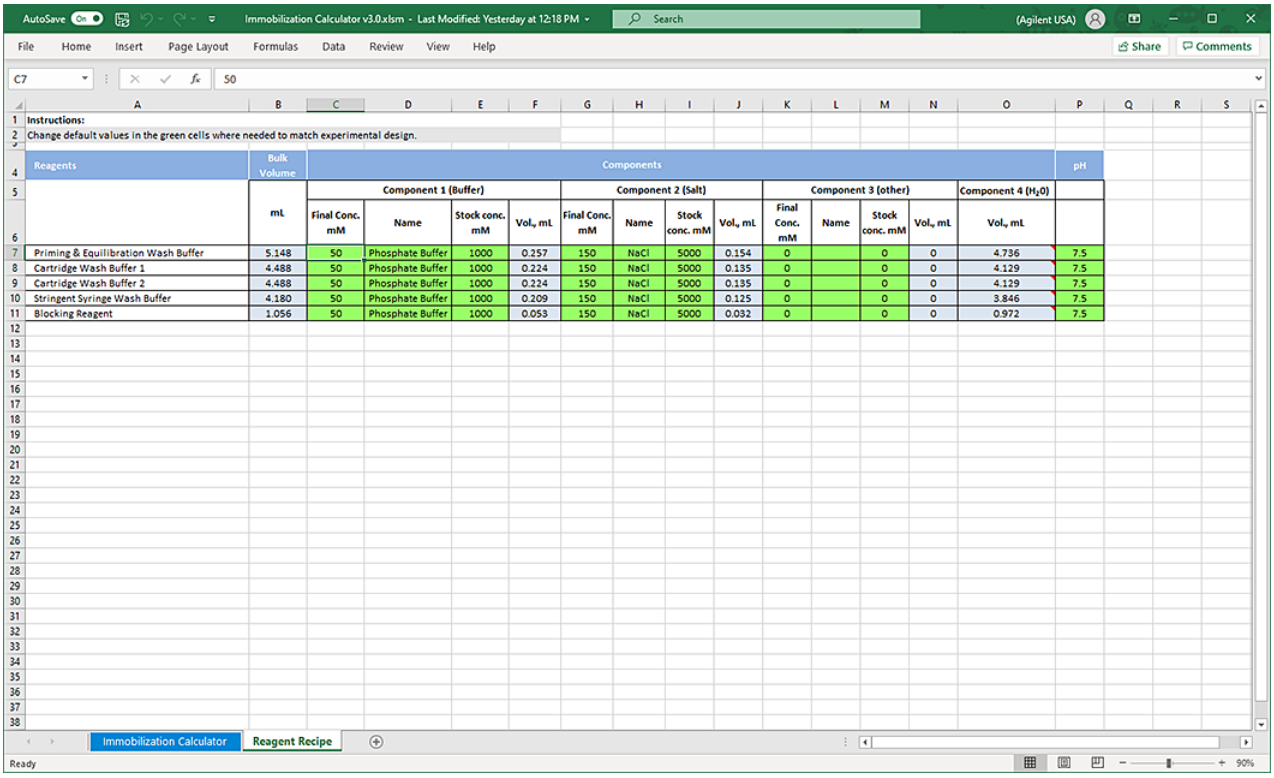


Figure Immobilization Reagent Recipe worksheet



Instructions:
Change default values in the green cells where needed to match experimental design.

Reagents	Bulk Volume	Components													pH
		Component 1 (Buffer)				Component 2 (Salt)				Component 3 (other)				Component 4 (H_2O)	
	mL	Final Conc. mM	Name	Stock conc. mM	Vol., mL	Final Conc. mM	Name	Stock conc. mM	Vol., mL	Final Conc. mM	Name	Stock conc. mM	Vol., mL	Vol., mL	
Priming & Equilibration Wash Buffer	5.148	50	Phosphate Buffer	1000	0.257	150	NaCl	5000	0.154	0		0	0	4.736	7.5
Cartridge Wash Buffer 1	4.488	50	Phosphate Buffer	1000	0.224	150	NaCl	5000	0.135	0		0	0	4.129	7.5
Cartridge Wash Buffer 2	4.488	50	Phosphate Buffer	1000	0.224	150	NaCl	5000	0.135	0		0	0	4.129	7.5
Stringent Syringe Wash Buffer	4.180	50	Phosphate Buffer	1000	0.209	150	NaCl	5000	0.125	0		0	0	3.846	7.5
Blocking Reagent	1.056	50	Phosphate Buffer	1000	0.053	150	NaCl	5000	0.032	0		0	0	0.972	7.5

Preparing the buffers

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the AssayMAP immobilization chemistry.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming & Equilibration Buffer (deck location 3)	Typically a buffered aqueous solution with neutral pH and physiologic salt concentration, for example, Phosphate-Buffered Saline (PBS), similar in composition to the buffer solution used to prepare the sample. *Note:* The Immobilization application also uses this buffer to wash the cartridges during the re-equilibration step.

Reagent (deck location)	Composition and comments
Cartridge Wash Buffer 1 (deck location 5)	A high-stringency buffer (for example, a neutral buffer with high NaCl) or a low-stringency buffer (for example, PBS). The Immobilization application allows the use of one or two wash solutions. The buffer selection depends on a number of factors. First, consider whether a blocking agent will be used. If so, one would typically use the low stringency solution for Cartridge Wash Buffer 1 and a high stringency wash buffer for Cartridge Wash Buffer 2, which would remove the loosely bound affinity ligand and blocking reagent from the resin bed. If a blocking reagent is not used, you would typically use a high-stringency wash buffer, such as PBS with high salt for Cartridge Wash 1 and use a low-stringency wash for Cartridge Wash Buffer 2.
Cartridge Wash Buffer 2 (deck location 6)	A high-stringency buffer (for example, a neutral buffer with high NaCl) or a low-stringency buffer (for example, PBS). The composition of this buffer is often dictated by the composition of Cartridge Wash Buffer 1. Cartridge Wash Buffer 2 will be replaced by the Priming & Equilibration Buffer if the Re-Equilibrate step is selected.
Stringent Wash Buffer (deck location 8)	The composition can vary widely depending on the experiment. Typically, this buffer would match the composition of the elution buffer that will be used during the Affinity Purification run, which usually follows the Immobilization run.
Blocking Reagent (deck location 9)	This reagent is most commonly used with SA-W cartridges in which case a free biotin solution is used to bind to the unbound streptavidin molecules on the surface of the resin. This blocks the streptavidin molecules that are not bound to the affinity ligand from binding to molecules present in the sample loaded on the cartridges during a subsequent Affinity Purification run using these cartridges.

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the samples at deck location 4 and cartridge positions in the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 203.

To dispense the solutions into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Priming & Equilibration Buffer into the labware to be placed at deck location 3.
3. Add the specified volume of Cartridge Wash Buffer 1 into the labware to be placed at deck location 5.
4. If using two wash buffers, add the specified volume of Cartridge Wash Buffer 2 into the labware to be placed at deck location 6.

5 Add the specified volume of Stringent Wash Buffer into the labware to be placed at deck location 8.

6 Add the specified volume of Blocking Reagent into the labware to be placed at deck location 9.

7 If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT To prevent evaporation, samples should be prepared immediately before running the protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to optimize the binding conditions (for example, pH).
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the sample composition

The optimal chemical environment for binding is generally similar for protein A, protein G, and streptavidin.

- Protein A and G resins bind selectively to antibodies.

  Examine the scientific literature for differences in their affinity for antibody subtypes from different species.
- Streptavidin resin binds selectively to biotinylated molecules.
- Protein A, protein G, and streptavidin are relatively unaffected by most sample components, including those present in complex protein mixtures.

What are optimal pH conditions?

One of the most important considerations for optimizing binding conditions is the pH of the sample, which should be near neutral pH. Both low (2 to 3) and high (10 to 11) pH ranges can prevent binding to protein A or protein G resins. The sample should generally be:

- *Protein A*. Greater than pH 6.
- *Protein G*. Greater than pH 4.

What sample components cause concerns?

Protein A, protein G, and streptavidin generally tolerate moderate levels of salt, non-ionic detergents, and mild denaturing reagents, such as urea, quite well. You should examine scientific literature for the known effects and tolerances of protein A, protein G, and streptavidin, keeping in mind that these tolerances may differ depending on the antibody species and subtype for protein A and protein G.

Does the antibody species and isotype in the sample match the cartridge binding specificity?

Protein A and protein G bind a wide variety of antibody subtypes and species.[1] Carefully consider the species and subtype of antibody when choosing between using an AssayMAP Protein A or Protein G cartridge for purification.

The antibodies that bind to protein G largely overlap the set that binds to protein A. While protein A is the industry standard for purification and titer determination of human therapeutic antibodies, protein G is the standard for purification of antibodies used as bioanalytical tools, primarily because many antibody subtypes are generated in species that bind poorly to protein A, for example, mouse IgG_1 and rat IgG_1.[2]

Determining the volume of sample to load

The AssayMAP Immobilization protocol permits loading up to 1000 µL of sample onto AssayMAP cartridges. For sample volumes > 250 µL, the protocol will iteratively load samples onto cartridges to stay within the maximum syringe volume (250 µL) of the Bravo 96AM Head.

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of the target molecule that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through. This value is dependent on the sample load flow rate.
- *Total binding capacity*. The maximum mass of the target molecule that can bind to the cartridge. This can be achieved only by loading significantly more of the target molecule than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity.

See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed information about the binding capacity for Protein A, Protein G, and Streptavidin cartridges.

What is the concentration of the affinity ligand in the sample?

If you know the approximate concentration of the target molecule in your sample and you are working within the quantitative binding range of the cartridge, you can determine the volume of sample to load as follows:

$$\text{µL sample to load} = \frac{\text{µg affinity ligand to bind}}{\text{µg/µL affinity ligand in the sample}}$$

How much affinity ligand should I immobilize?

The amount of affinity ligand to immobilize depends on the amount of target. Use at least a 5-fold molar excess compared to the amount of target to be captured. A low molar excess of affinity ligand (approximately 5-fold) requires a very slow loading flow rate (approximately 2 µL/min). As the molar excess increases so too can the loading flow rate. The exact molar excess and the flow rate should be determined empirical as they depend on many factors such as the strength of the affinity interaction and the diffusion rate of the target molecule.

Preparing the sample plates

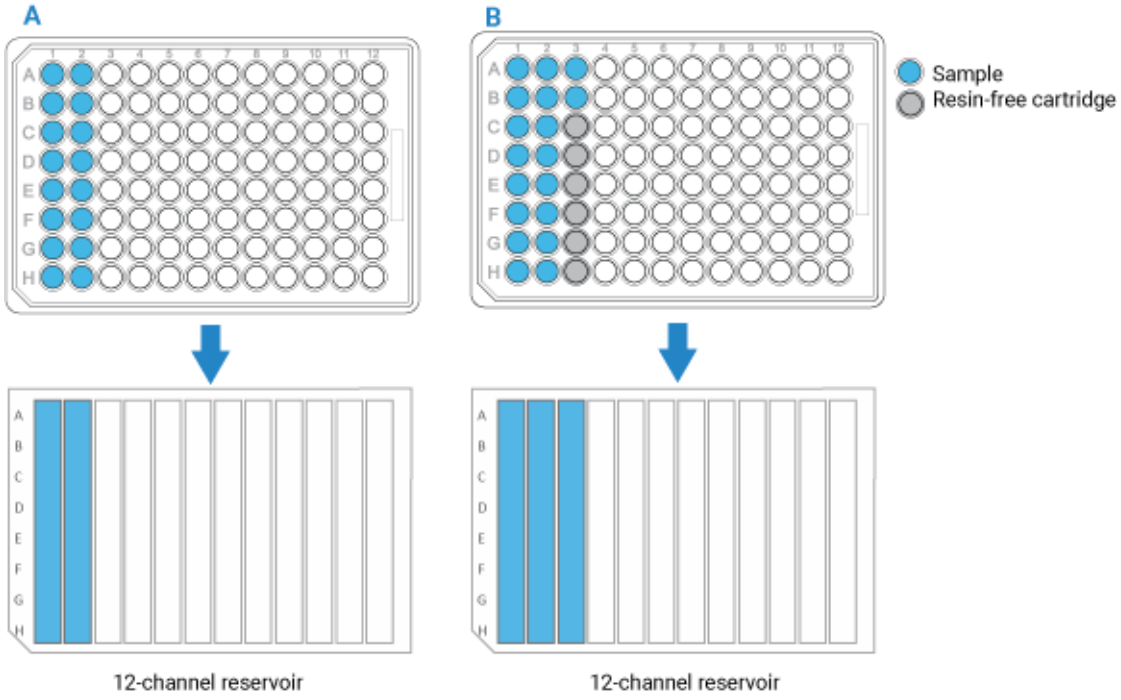
Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.

  The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based on the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use AssayMAP Resin-Free cartridges to fill the empty well positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash steps.

Figure Example of sample microplate and reservoir layout: A) Multiple of 8 samples, and B) Not a multiple of 8



See "Labware" on page 197 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION

A small volume excess is required in all labware types to ensure proper volume transfer.

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

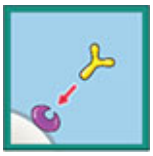
The reagent volume calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol



The Immobilization protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- If applicable (for example, using SA-W cartridges), blocks free binding sites.
- Removes non-specific binding molecules from the cartridges.

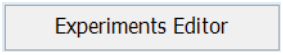
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the Cartridge & Tip Seating Station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 199.
- Prepare the samples. See "Preparing the samples" on page 203.

- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the cartridge bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

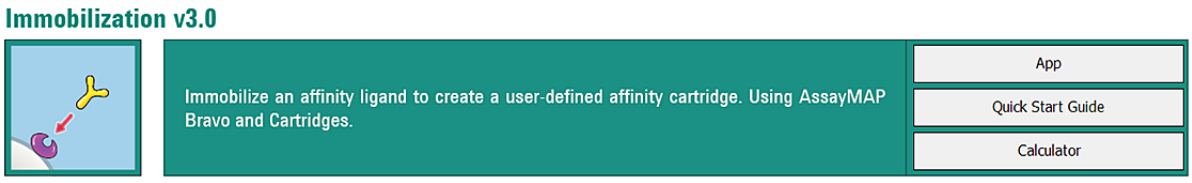
IMPORTANT Do not allow wetted cartridges to dry out. Agilent Technologies does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 196.

Setting up the protocol

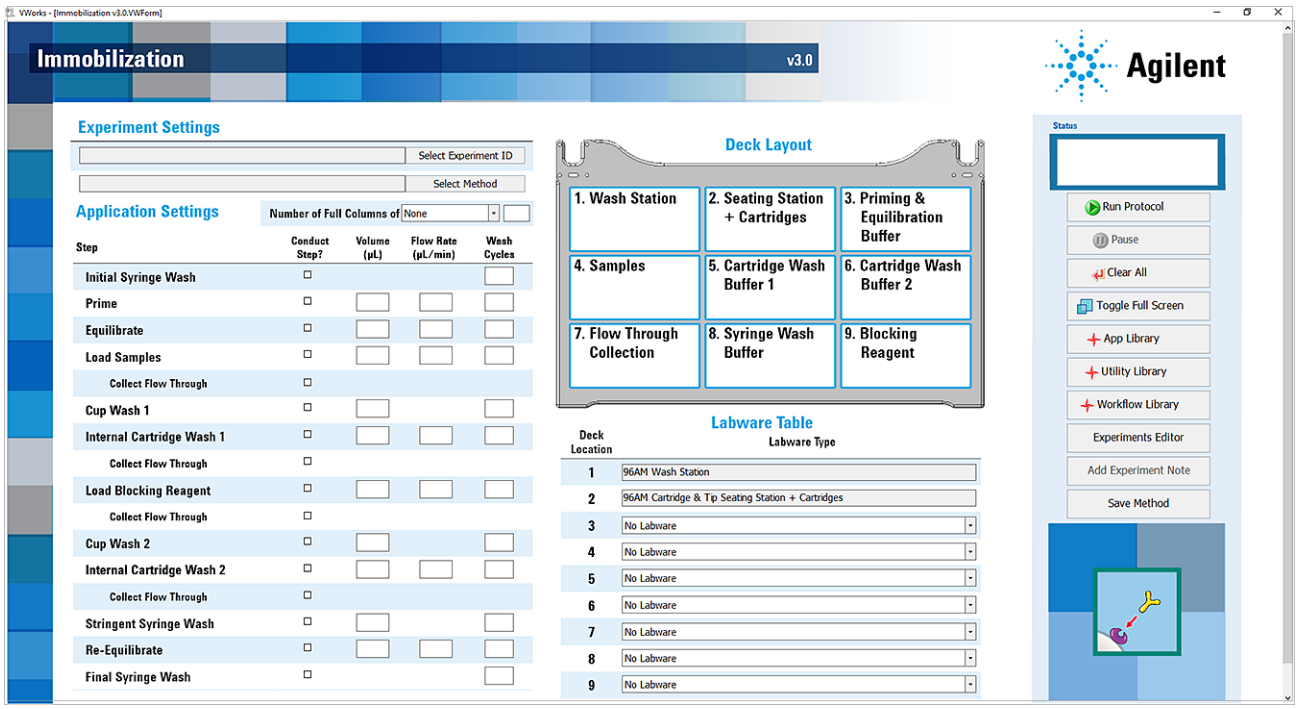
Before starting the protocol, make sure the appropriate selections and values are specified in the Immobilization application.

To set up the protocol:

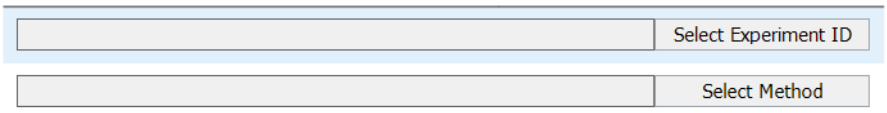
1. If not already open, open the **App Library**.
2. Locate **Immobilization**, and then click **App**.



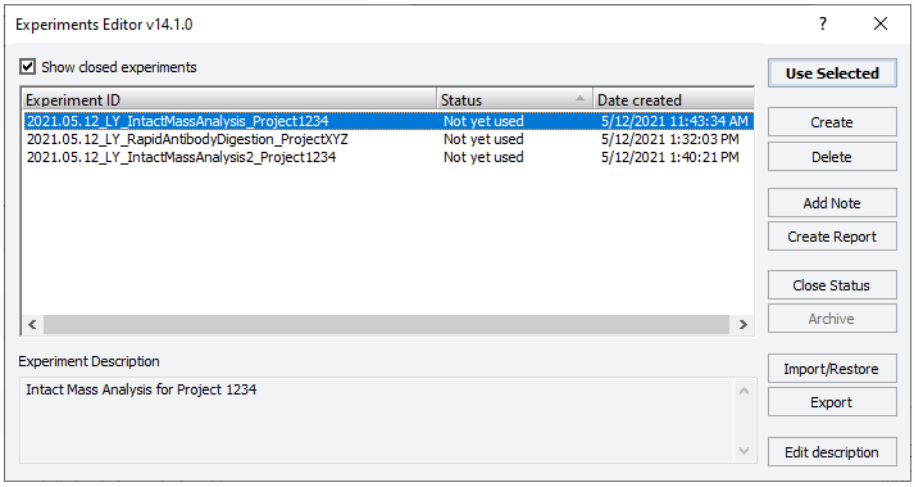
The Immobilization application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 214.
- To modify the method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:

a Select the cartridge size from the list:
- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION

If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.

CAUTION

If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.

IMPORTANT

Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 216.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10
Equilibrate	Aspirates Equilibration Buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates samples (deck location 4) into the syringes, and then dispenses them through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–1000	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the sample flow-through to waste in the wash station (deck location 1).	–	–	–	–
Cup Wash 1	Rinses the cartridge cups with Cartridge Wash Buffer 1 (deck location 5), and then discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Internal Cartridge Wash 1	Aspirates Cartridge Wash Buffer 1 (deck location 5) into the syringes, and then dispenses it through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash 1 flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the Internal Cartridge Wash 1 flow-through into the wash station (deck location 1).	–	–	–	–
Load Blocking Reagent	Aspirates Blocking Reagent (deck location 9) into the syringes, and then dispenses it through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	50	5	3
		25 µL:	250	5	3
		Range:	0–250	0.1–500	0–10
Collect Flow Through	If selected, collects the Blocking Reagent flow-through in the Flow Through Collection plate (deck Location 7). If not selected, discards the Blocking Reagent flow-through into the wash station (deck location 1).	–	–	–	–
Cup Wash 2	Rinses the cartridge cups with Cartridge Wash Buffer 2 (deck location 6) and discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 2	Aspirates Cartridge Wash Buffer 2 (deck location 6) into the syringes, and then dispenses it through the cartridges into the Flow Through Collection plate (deck location 7) or into the wash station (deck location 1).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects Internal Cartridge Wash 2 flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards Internal Cartridge Wash 2 flow-through into the wash station (deck location 1).	–	–	–	–
Stringent Syringe Wash	Aspirates Syringe Wash Buffer (deck location 8) into the syringes, and then discards the liquid into the wash station (deck location 1).	5 µL:	50	–	2
		25 µL:	50	–	2
		Range:	0–250	–	0–10
Re-Equilibrate	Aspirates Equilibration Buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 217.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 230.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT

The protocol will display an error message if cartridges are missing.

Starting the protocol run

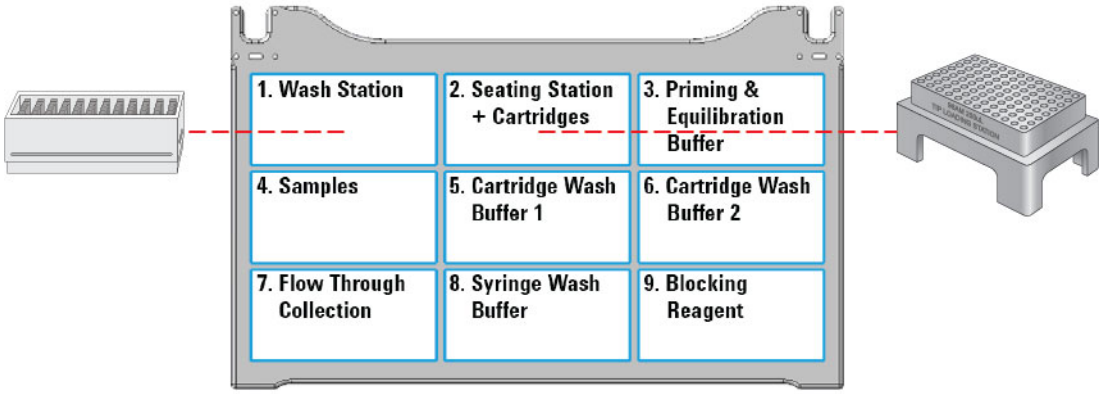
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

Note: The Greiner PCR plates are not compatible with the 25 µL cartridges at deck location 7.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

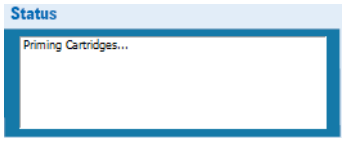
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

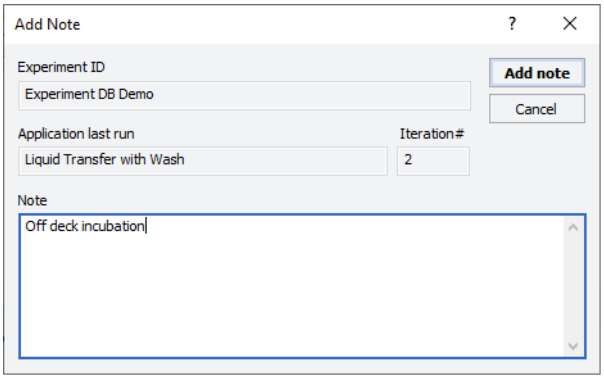
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

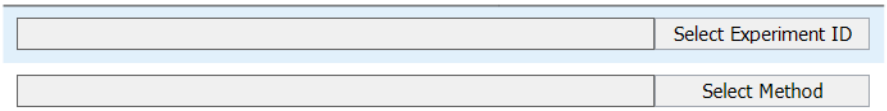
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

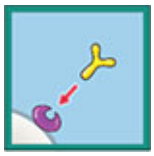
f Click Run Protocol to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes



This topic explains the following:

- Each step of the protocol so that you can optimize the Immobilization protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the packed resin bed and properly wets the surface of the resin. In preparation for priming, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP affinity purification cartridges (PA-W, SA-W, and PG-W) typically used with this application contain affinity ligands (proteins) covalently immobilized onto resin. These cartridge types should be primed with aqueous buffers containing no or low amounts of organic solvent or protein denaturants. Because the Equilibration Buffer is drawn from the same reservoir as the Priming Buffer, buffers that favor analyte binding with minimal non-specific binding should be used for both priming and equilibration. This step is selected by default. **Volume (µL)**. The default volume should be sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridges: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default value diminishes the ability to effectively remove entrained air from the resin bed. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate step tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the sample loading begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates sample into the syringes, and then performs an external syringe wash at the wash station to remove any sample remaining on the outside of the probes before mounting the cartridges. The samples are dispensed through the cartridges into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove sample on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. The protocol accommodates sample volumes up to 1000 µL to be dispensed through the cartridges. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. Each syringe has a maximum capacity of 250 µL. When sample volumes are greater than 250 µL, the protocol will iteratively load samples onto cartridges. To determine the number and volume of the iterative load steps, the protocol uses the following formulas: • *# of times to load* = total sample volume/250, where # times to load is rounded up to nearest integer • *volume of each load* = sample volume/# of times to load For example, if the total sample volume is 900 µL, then: *# times to load* = 900/250 = 3.6, which is rounded up to 4 *volume of each load* = 900/4 = 225 If Collect Flow Through is selected for the Load Samples step, be sure that the Flow Through Collection plate has sufficient maximum well capacity. For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. **IMPORTANT** Be sure to include the recommended labware-dependent volume overage to prevent air from entering the cartridge. For more information, see "Preparing the sample plates" on page 205. To determine the volume of sample to load, see "Determining the volume of sample to load" on page 204. This step is selected by default.

Protocol step	Guidelines and notes
	Volume (µL): The volume of sample to load should be balanced with the sample concentration and the mass capacity of the cartridge. The lower the sample volume, the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. Large sample volumes (> 250 µL) may require slightly more excess sample due to evaporation. • Default: 100 • Practical: 10–1000 • Range: 0–1000 *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min)**. The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than 5 µL/min may not significantly increase analyte binding, but this is highly dependent on the molar ratio of the capture ligand compared to the target molecule. For examples of cases with flow rates of less than 5 µL/min, see Agilent app notes 5991-9010EN and 5991-8445EN in the "Reference library" on page 234. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed in the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step is dispensed directly into the wash station. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is selected by default.

Protocol step	Guidelines and notes
Cup Wash 1	This step removes the residual sample liquid that may remain above the resin bed after the Load Samples step. The Cup Wash 1 step aspirates Cartridge Wash Buffer 1 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the wash station, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 1	This step uses Cartridge Wash Buffer 1 to wash non-specifically bound molecules from the resin bed. In preparation for Internal Cartridge Wash 1, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Cartridge Wash Buffer 1 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 1 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. If the Load Samples step is selected, the first 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) of Cartridge Wash Buffer 1 is dispensed as a sample chase at the Load Samples flow rate. Next, the Internal Cartridge Wash volume minus the chase volume is dispensed at the Internal Cartridge Wash flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the flow-through from Internal Cartridge Wash 1 is dispensed in the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from Internal Cartridge Wash 1 is dispensed at the wash station. This step is not selected by default.
Load Blocking Reagent	This step allows a reagent of defined composition to be flowed through the cartridge after ligand immobilization to help minimize non-specific binding in subsequent target purification steps. The Load Blocking Reagent step may be especially helpful when a sub-saturating amount of ligand is bound to the resin in the cartridge. In preparation for blocking, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Blocking Reagent is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Load Blocking Reagent step aspirates Blocking Reagent into the syringes, performs an external syringe wash at the wash station, mounts the cartridges, dispenses the Blocking Reagent through the cartridges at the specified flow rate to either the Flow Through Collection plate or the wash station. An external cartridge wash is performed at the wash station to remove any blocking reagent on the outside of the cartridge. The cartridges are parked at the seating station and the syringes are washed at the wash station. Select the Load Blocking Reagent step to minimize to non-specific binding during subsequent target purification steps. This step is selected by default.

Protocol step	Guidelines and notes
Load Blocking Reagent (continued)	**Volume (µL)**. The volume of Blocking Reagent to aspirate into the syringes and dispense through the cartridges. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Load Blocking Reagent tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default will likely have no benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, which may result in incomplete blocking. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the liquid eluted during the Load Blocking Reagent step is dispensed into the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Blocking Reagent step is dispensed into the wash station. Select this step if you want to collect the Blocking Reagent flow-through. This step is not selected by default.

Protocol step	Guidelines and notes
Cup Wash 2	This step removes the residual buffer that may remain above the resin bed after the Load Blocking Reagent step. This step aspirates Cartridge Wash Buffer 2 and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the previous step is aspirated from the cartridge cups. Any cartridges that stuck to the probes during the cup wash are removed at the seating station, and then the liquid in the syringes is dispensed into the wash station. The syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. A volume less than the default may be insufficient for cup washing, while a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash 2 tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 2	This step uses Cartridge Wash Buffer 2 to wash non-specifically bound molecules and Blocking Reagent from the resin bed. In preparation for Internal Cartridge Wash 2, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Cartridge Wash Buffer 2 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 2 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges at the specified flow rate into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer from the previous step on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but will also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the liquid eluted during Internal Cartridge Wash 2 is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through is dispensed into the wash station. Select this step if you want to collect the flow-through generated during Internal Cartridge Wash 2. This step is not selected by default.
Stringent Syringe Wash	This step cleans the syringes with the Stringent Syringe Wash Buffer. The Stringent Syringe Wash step aspirates the Stringent Syringe Wash Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the wash station. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume are unlikely to improve the syringe cleaning but will increase the run time. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash cycle**. A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Re-Equilibrate	This step conditions the resin and immobilized ligand to prepare them for the next step in the workflow, for example, running the Affinity Purification protocol. In preparation for re-equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Re-Equilibration step aspirates the Equilibration Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. Do not allow cartridges to dry out. After re-equilibration, use immediately for affinity purification or store them short term in the receiver plate containing 200 µL of Equilibration Buffer per well. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully re-equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Re-Equilibrate tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads in the cartridge resin bed. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, the 10 µL of elution buffer will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. *Note:* In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Immobilization protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	2	Parks any cartridges that may have been mounted on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes the specified number of times.

Protocol step	Head moves to deck location...	Action
Prime	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then washes the exterior of the syringe probes.
	3	Aspirates 10 µL of Priming & Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	3	Aspirates the Priming Buffer.
	2	Mounts the cartridges on the head.
	1	Dispenses the Priming & Equilibration Buffer through the cartridges into the wash station, and then washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then washes the exterior of the syringe probes.
	3	Aspirates 10 µL of Priming & Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	3	Aspirates the Priming & Equilibration Buffer.
	2	Mounts the cartridges on the head.
	1	Dispenses the buffer through the cartridges to the wash station.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Load Samples	4	Aspirates samples into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	7	Dispenses sample through the cartridges to load sample. Collects flow-through in the Flow Through Collection plate.
	1	Washes the cartridge exteriors at the wash station.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash 1	5	Aspirates Cartridge Wash Buffer 1 into the syringes.
	2	Performs the cup wash and exercise the cartridges off task.
	1	Dispenses buffer into the wash station.
	1	Washes the syringes.
Internal Cartridge Wash 1	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys.
	5	Aspirates 10 µL of Cartridge Wash Buffer for the cartridge air-gap prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	5	Aspirates Cartridge Wash Buffer 1 into the syringes.
	2	Mounts the cartridges on the head.
	1	Dispenses 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) Cartridge Wash Buffer 1 through the cartridges at the Load Samples flow rate for the sample chase step.
	1	Dispenses the remaining Cartridge Wash Buffer 1 through the cartridges at the Internal Cartridge Wash 1 flow rate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes at the wash station.

Protocol step	Head moves to deck location...	Action
Load Blocking Reagent	9	Aspirates Blocking Reagent into syringes.
	1	Washes exterior of probes at the wash station.
	2	Mounts the cartridges on the head.
	1	Dispenses Blocking Reagent through the cartridges.
	1	Washes the cartridge exteriors at the wash station.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes at the wash station.
Cup Wash 2	6	Aspirates Cartridge Wash Buffer 2 into the syringes.
	2	Performs the cup wash and exercise the cartridges off task.
	1	Dispenses buffer into the wash station.
	1	Washes the syringes at the wash station.
Internal Cartridge Wash 2	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then washes the exterior of the syringe probes.
	6	Aspirates 10 µL of Cartridge Wash Buffer 2 for the cartridge air-gap prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates Cartridge Wash Buffer 2 into the syringes.
	2	Mounts the cartridges on the head.
	1	Dispenses Cartridge Wash Buffer 2 through the cartridges.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes at the wash station.
Stringent Syringe Wash	8	Aspirates the Stringent Syringe Wash Buffer.
	1	Dispenses the buffer into the wash station.
	1	Washes the syringes at the wash station.

Protocol step	Head moves to deck location...	Action
Re-Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then washes the exterior of the syringe probes.
	3	Aspirates 10 µL of Priming & Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	3	Aspirate the Priming & Equilibration Buffer.
	2	Mounts the cartridges.
	1	Dispenses buffer through the cartridges into the wash station.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes at the wash station.
Final Syringe Wash	2	Moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys.
	1	Washes the syringes at the wash station.

Reference library

1 Terry, M.P. in Handbook of Affinity Chromatography, Second Edition. (ed. D.S. Hage), 367-397 (CRC Press, 2005).

2 PROTEUS Protein G Antibody Purification Handbook, Mini & Midi spin columns, Bio-Rad Laboratories, Inc 2013.

3 Wu, S., Shen, M., Murphy, S. & Van Den Heuvel, Z., An Integrated Workflow for Intact and Subunits of Monoclonal Antibody Accurate Mass Measurements, Agilent Application Note 5991-8445EN, March 2018

4 Han, J., Van Den Heuvel & Murphy, S., A streamlined drug-to-antibody ratio determination workflow for intact and deglycosylated antibody-drug conjugates, Agilent Application Note 5991-9010EN, September 2019

See the Agilent AssayMAP Bravo Citation Index for published papers that used the AssayMAP Bravo Platform.

8 In-Solution Digestion: Multi-Plate v2.0 User Guide

This chapter contains the following topics:

App description

In-Solution Digestion: Multi-Plate v2.0. This application enables automated digestion of 1 to 384 protein samples in up to four 96-well microplates in a single run.

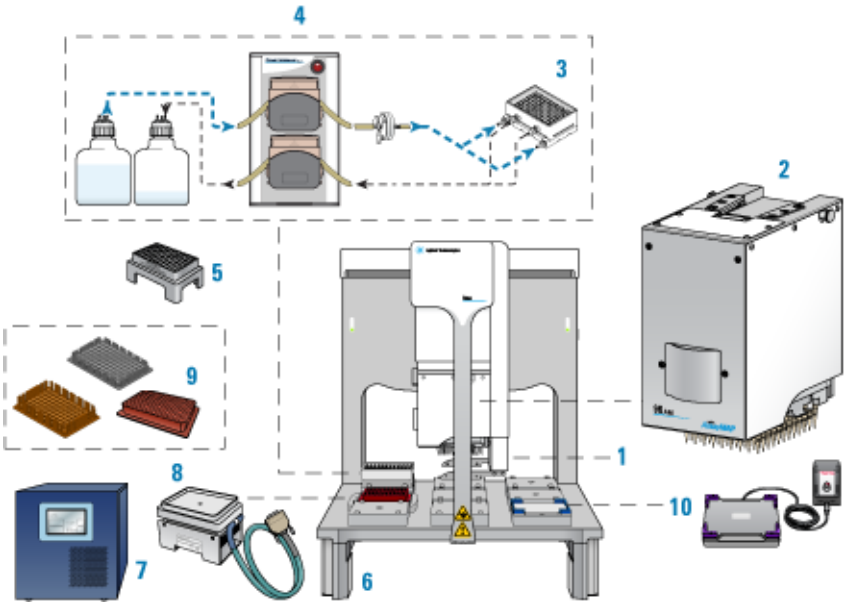
Before you start

This topic lists the required hardware, software, AssayMAP Starter Kit, labware, and reagents for running the In-Solution Digestion: Multi-Plate protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which is required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	Peltier Thermal Station with STC controller
8	Custom plate nest
9	Thermal plate insert for efficient heat transfer on the Peltier Thermal Station
10	Orbital Shaking Station with Control Unit

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

Labware and starter kits

The In-Solution Digestion protocol works with a broad range of user-supplied reagents for denaturation, reduction, alkylation, and proteolysis steps. This protocol requires a specific set of labware that can be sourced from various vendors.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Labware

The following table provides a complete list of labware options and the corresponding deck locations.

The following figures show the nine Bravo deck locations for labware.

Figure Reagent Plate Setup labware locations on the Bravo deck (top view)

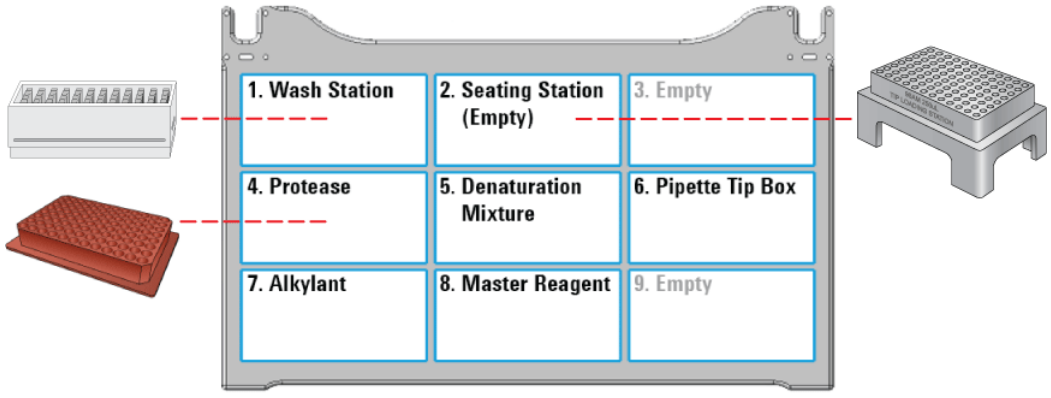
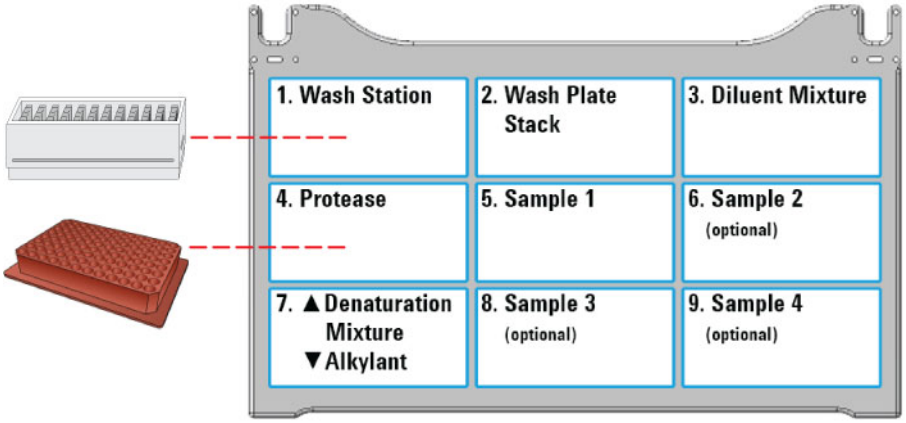


Figure In-Solution Digestion labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
Reagent Plate Setup labware		
• 96 Red PCR Insert + 96 Eppendorf 30129300, PCR, Full Skirt	Agilent insert (provided) and Eppendorf 30129300	4
• 96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	5, 7
• 250-µL pipette tips	Agilent 19477-002	6
• 96 ABgene 1127, Deep Well, Square Well, Round Bottom	ABgene AB-1127	8
In-Solution Digestion labware		
• 96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	2, 5– 9
• 96 ABgene 1127, Deep Well, Square Well, Round Bottom	ABgene AB-1127	3
• 96 Red PCR Insert + 96 Eppendorf 30129300, PCR, Full Skirt	Agilent insert (provided) and Eppendorf 30129300	4
*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.		

Starter kits

Agilent offers starter kits for performing In-Solution Digestion and Peptide Cleanup, as these two applications are often performed sequentially. The following table lists the two starter kits that are available for In-Solution Digestion and Peptide Cleanup. Each starter kit contains both cartridges and labware.

Kit	Part number
AssayMAP Digestion and RP-S Cleanup Starter Kit— Contains 96 reversed-phase (RP-S) cartridges and starter kit labware for In-Solution Digestion and Peptide Cleanup.	G5496-60034
AssayMAP Digestion and C18 Cleanup Starter Kit— Contains 96 C18 cartridges and starter kit labware for In-Solution Digestion and Peptide Cleanup.	G5496-60013

For more information on the cartridges, see the "AssayMAP cartridges" on page 358 in the Peptide Cleanup 4.0 User Guide.

Starter kit labware

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

The following table lists labware that are included in the starter kits (G5496-60034 and G5496-60013).

Note: Additional labware are included in the starter kits but are required for the Peptide Cleanup application only. For more information, see "Peptide Cleanup v4.0 User Guide" on page 355.

Labware	Part number
1.2 mL Deep-Well PolyPro Clear Plates (qty 2)	ABgene AB-1127
96-Well U-Bottom PolyPro White Plates (qty 11)	Greiner 650207
96-Well PCR Plates (qty 3)	Eppendorf 30129300
12-Column Low-Profile Reservoirs (qty 4)	Agilent 201280-100
96-Well Round-Bottom, Clear Plates (qty 2)	Greiner 650201
250-µL Pipette Tips	Agilent 19477-002

For details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Reagents

The volume, type, and concentration of reagents required to prepare for the In-Solution Digestion: Multi-Plate protocol depends on a combination of factors, including specific chemistry requirements, the number of samples to process, and volumes and concentrations of reagents necessary to conduct denaturation, reduction, alkylation, and proteolysis. The In-Solution Digestion Reagent Volume Calculator manages this complexity by preparing a reaction summary, optimized solution recipes, and microplate layouts for all master reagents based on your input values.

Consult published literature for reagent recommendations for sample and surface chemistry combinations. See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP In-Solution Digestion: Multi-Plate application.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 241.

Preparing the solutions

The In-Solution Digestion: Multi-Plate protocol accommodates a wide range of digestion chemistries so that previously optimized conditions can be easily transferred to this automated platform. Therefore, the reagents described here are categories of reagents used in the assay rather than specific recommendations.

Solutions required for the protocol

Table Required solutions

Solutions	Number of plates required	Description
Syringe Wash Buffer	4	This is an optional solution used to solubilize and wash away protein that may have adsorbed onto the probes and syringes. The use of 50 mM NaOH has been found to be a suitable stringent syringe wash solution for several proteins. However, other protein-solubilizing solutions may be used depending on the specific protein sample inputs. Use the Single Liquid Addition utility (Single Liquid Addition v2.0 User Guide) to transfer 300 µL per well of the wash solution to the four Greiner 96-Well Round Bottom, White Plates that will be stacked on deck location 2 by the Plate Stacking protocol before running the In-Solution Digestion protocol. During the protocol run, these plates serve two purposes. The plates are moved on top of the sample plates and serve as lids to protect the samples from light during the alkylation step of the protocol. The plates are also used as reservoirs for the syringe wash solution which is used to wash the syringes to prevent carry-over between sample loading steps.

Solutions	Number of plates required	Description
Master Reagents	1	The three master mix reagents (Denaturation mixture, alkylant, and protease) are placed in the reagent setup plate which serves as the source plate for distributing these reagents into the plates used during the In-Solution Digestion: Multi-Plate protocol. Use the Reagent Plate Setup protocol to dispense these three reagents to dedicated reagent plates. Fill an ABgene 1.2 mL Deep-Well Plate with the Master Reagent. See "Setting up the Reagent Plate Setup protocol" on page 255.
Diluent Mixture	1	This solution is used to dilute the sample following denaturation, reduction, alkylation and before enzyme addition. The solution typically contains deionized water, 1.0 M Tris at pH 8.1, and 0.5 M TCEP. This reagent is placed in an ABgene 1.2-ml deep-well plate which is placed at deck location 3 during the In-Solution Digestion protocol run. The volume per well required for the Diluent Mixture and the composition of the solution is determined using the Reagent Volume Calculator.

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for In-Solution Digestion: Multi-Plate

The Reagent Volume Calculator is a Microsoft Excel file that contains the following:

- *Reaction Setup worksheet*. You to enter information about the samples, denaturant and reductant, alkylant, dilution, and digestion. The calculator determines the concentrations and volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

  Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.
- *Reagent Prep worksheet*. This sheet displays the volumes and composition of the reagents required for the in-solution assay based on the input provided in the Reaction Setup worksheet.

  The Reaction Prep worksheet consists of four tables: Denaturation Mixture, Alkylant, Protease, and Diluent Mixture

  If you are using more denaturant than what is required, you can enter the larger mass in the Denaturation Mixer table.

  The Master Reagent requires iodoacetamide powder. If you are using more than what is required, you can enter the larger mass in the Alkylant table.

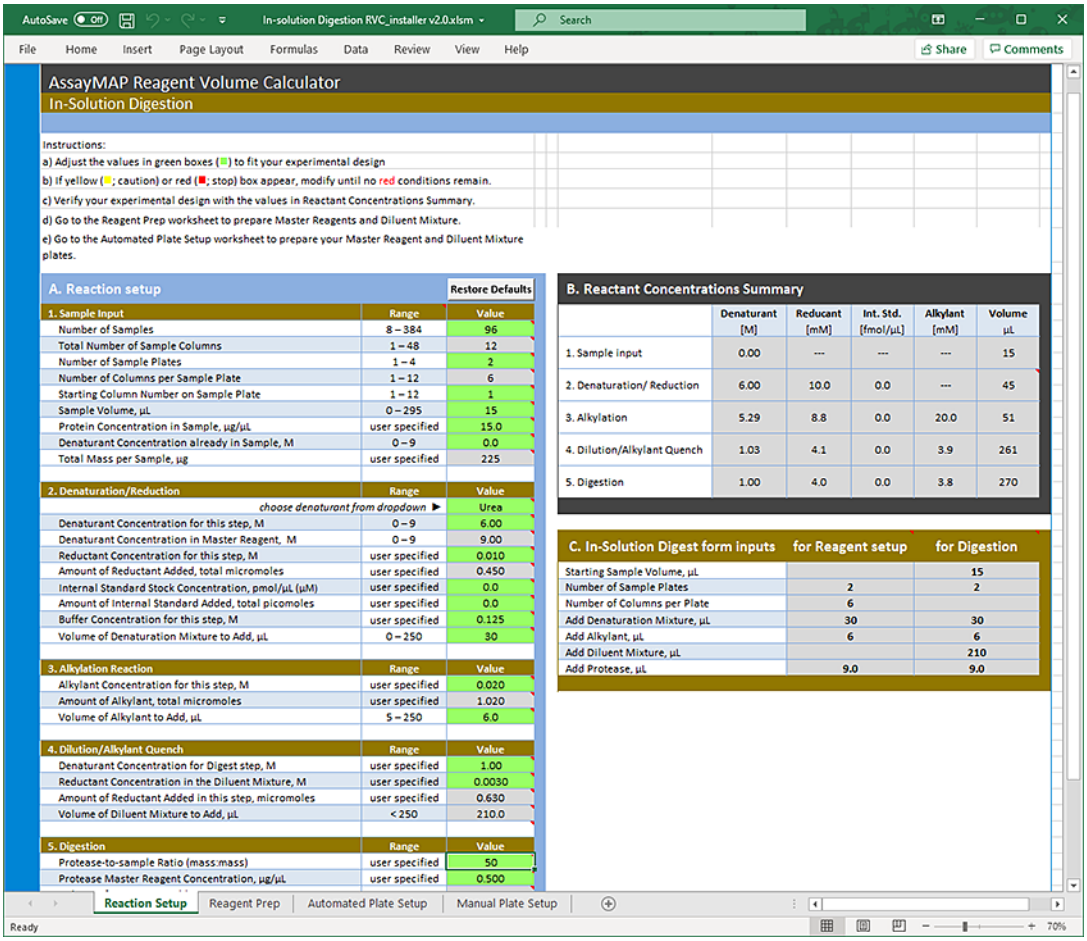
- *Automated Plate Setup worksheet*. This sheet displays the layout of the Master Reagent plate and the Diluent Mixture plate, including the volumes of the reagents that must be transferred into the designated wells when running the Reagent Plate Setup protocol.
- *Manual Plate Setup worksheet*. This sheet displays the layout of the Denaturation Mixture plate, Alkylant plate, Protease plate, and Diluent Mixture plate, including the volumes of the reagents required for the plates used in the In-Solution Digestion protocol in case you prefer to manually pipette these reagents into the plates rather than use the Reagent Plate Setup protocol.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click **Restore Defaults**.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.



Reagent Volume Calculator Reaction Setup fields

The following tables describe the fields in the Reaction Setup worksheet.

Reaction Setup: Sample Input area

Field	Description
Number of Samples	The number of samples you want to process. The number of samples should be a multiple of 8. *Note:* If the number of samples is not a multiple of 8, excess reagent consumption will occur. See Total Number of Sample Columns. Range: 8–384 Default: 96
Total Number of Sample Columns	*Read only*. The calculator converts the Number of Samples to the Total Number of Sample Columns by rounding up to the nearest multiple of 8. Range: 1–48 Default: 12

Field	Description
Number of Sample Plates	The number of labware containing the samples you want to process. To minimize the volume of reagent consumed, specify a greater number of sample plates where fewer wells are used in each plate. This requires fewer syringes during the liquid transfer, which minimizes reagent consumption. To minimize the number of labware used, specify fewer number of sample plates. For example, place eight columns of samples on one plate. Because more syringes are used during the liquid transfer, more reagents are consumed. To assess which scenario works best, use the Reagent Prep worksheet. Range: 1–4 Default: 2
Number of Columns per Sample Plate	*Read only*. The number of columns that occupy each sample plate. The value is calculated using the Total Number of Sample Columns and the Number of Sample Plates. Range: 1–12 Default: 6
Starting Column Number on Sample Plate	The column at which the samples begin in the sample plate. *Note:* The samples can begin in any column in the sample plate. Make sure the columns in the plates are contiguous. You cannot skip a column. If you have multiple sample plates, make sure the samples begins at the same column across all the plates. Range: 1–12 Default: 1
Sample Volume, µL	The volume of the samples in each well. **IMPORTANT** Make sure the labware you are using can accommodate the sample volume you specify, plus the additional volume that will be added during the course of the run. You can start with the default value, 15 µL, because it satisfies a typical set of reaction parameters. Range: 0–270 Default: 15
Protein Concentration in Sample, µg/µL	The concentration of the protein in the sample. The In-Solution Digestion protocol can accommodate a wide range of concentrations, which is largely dependent on downstream needs. Range: Varies Default: 15

Field	Description
Concentration of Denaturant in Sample, Molar	The concentration of urea or guanidine already present in the sample. *Note:* The acceptable range differs for each denaturant. In addition, the value you specify influences calculations for the Denaturation Mixture. Range: 0–8 for guanidine; 0–9 for urea Default: 0.0
Total Mass per Sample, µg	*Read only*. The mass of the sample. The value is calculated using the Sample Volume and Protein Concentration. Range: Varies Default: 225

Reaction Setup: Denaturant/Reduction area

2. Denaturation/Reduction	Range	Value
choose denaturant from dropdown ▶		
Denaturant Concentration for this step, M	0 – 9	6.00
Denaturant Concentration in Master Reagent, M	0 – 9	9.00
Reductant Concentration for this step, M	user specified	0.010
Amount of Reductant Added, total micromoles	user specified	0.450
Internal Standard Stock Concentration, pmol/µL (µM)	user specified	0.0
Amount of Internal Standard Added, total picomoles	user specified	0.0
Buffer Concentration for this step, M	user specified	0.125
Volume of Denaturation Mixture to Add, µL	0 – 250	30

Field	Description
Choose denaturant from dropdown	The denaturant you want to use. Select either Guanidine or Urea.
Denaturant Concentration for this step, Molar	The concentration of guanidine or urea that you want to use in the denaturation step. To skip denaturation, enter 0. **IMPORTANT** If you are performing the Denaturation step, make sure the concentration is within the range for the denaturant. The permissible range ensures stability during the protocol run. Range: 0–8 for guanidine; 0–9 for urea Default: 6.00
Denaturant Concentration in Master Reagent, Molar	*Read only*. The concentration of guanidine or urea in the Master Reagent. To adjust the calculated value, increase or decrease the Volume of Denaturation Mixture to Add. For example, increase the Volume of Denaturation Mixture to Add to decrease the Denaturant Concentration in Master Reagent. Range: 0–8 for guanidine; 0–9 for urea Default: 9.00

Field	Description
Reductant Concentration for this step, Molar	The concentration of the reductant for the Denaturation step. To skip reduction, enter 0. Range: 0–0.100 Default: 0.010
Amount of Reductant, total micromoles	*Read only*. The micromoles of reductant. The value is calculated using the Reductant Concentration and the Denaturation Mixture volume. Range: Varies Default: 0.450
Internal Standard Stock Concentration, pmol/µL	*Optional*. The concentration of the internal standard stock solution you want to add to the Denaturation Mixture. Range: Varies Default: 0.0
Amount of Internal Standard in reaction, total picomoles	The total number of picomoles of the internal standard you want to include in the reaction. The value is converted to a final concentration at the indicated reaction steps (displayed in B. Reactant Concentrations Summary). Range: Varies Default: 0.0
Buffer Concentration for this step, Molar	The concentration of the pH buffer present during the denaturation step. *Note:* Values below 0.1 M have not been tested. Values above 0.35 M are out of range for the solutions used to prepare Master Reagents. Range: 0.100–0.350 Default: 0.125
Volume of Denaturation Mixture to add, µL	The volume of Denaturation Mixture that will be added into the sample. This mixture may include denaturant, reductant, internal standard, and pH buffer as defined by the experiment. Range: 0–270 Default: 30

Table Reaction Setup: Alkylation Reaction area

3. Alkylation Reaction	Range	Value
Alkylant Concentration for this step, M	user specified	0.020
Amount of Alkylant, total micromoles	user specified	1.020
Volume of Alkylant to Add, µL	5 – 250	6.0

Field	Description
Alkylant Concentration for this step, Molar	The concentration of alkylant desired at this step. Range: Varies Default: 0.020

Field	Description
Amount of Alkylant, total micromoles	*Read only*. The number of micromoles of the alkylant. The value is calculated from the concentration and volume added to the reaction, and the final concentration at the indicated steps is displayed in the B. Reactant Concentrations Summary table. Range: Varies Default: 1.020
Volume of Alkylant to add, µL	The volume of alkylant to add. Using the default or minimum volume (5 uL) minimizes unnecessary dilution of the reaction and provides greatest flexibility in the volumes of other reactants. Range: 5–250 Default: 6.0

Reaction Setup: Dilution/Alkylant Quench area

4. Dilution/Alkylant Quench	Range	Value
Denaturant Concentration for Digest step, M	user specified	1.00
Reductant Concentration in the Diluent Mixture, M	user specified	0.0030
Amount of Reductant Added in this step, micromoles	user specified	0.630
Volume of Diluent Mixture to Add, µL	< 250	210.0

Field	Description
Denaturant Concentration for Digest step, Molar	The concentration of guanidine or urea in the digestion step. This value determines the volume of Diluent Mixture. If the sample does not contain any denaturant, enter 0. If the Denaturant Concentration is 0, the software will allow you to manually enter a volume in the Volume of Diluent Mixture if NO Denaturant field (the last row in the 4. Dilution/Alkylation Quench area) to control the buffering capacity in the digestion reaction. Range: Varies Default: 1.00
Reductant Concentration in the Diluent Mixture, Molar	The concentration of reductant necessary for quenching the alkylant. The final concentrations of reductant and alkylant at the indicated reaction steps are summarized in the B. Reactant Concentrations Summary table. Range: Varies Default: 0.0030

Field	Description
Amount of Reductant added in this step, micromoles	*Read only*. The number of micromoles of reductant that is added during this step. The value is calculated using the Reductant Concentration and Volume of Diluent Mixture. Range: Varies Default: 0.630
Volume of Diluent Mixture to add, µL	*Read only*. The volume of the Diluent Mixture you want to add. The value is calculated using many of the values you supplied in the table. Range: < 250 Default: 210.0
Volume of Diluent Mixture if NO denaturant, µL	The volume of Diluent Mixture to add if Denaturant Concentration for Digest Step is 0. The Diluent Mixture contains additional reductant, if added, and pH buffer. This field appears only if you entered 0 for Denaturant Concentration for Digest Step (the first field in the 4. Dilution/Alkylation Quench area).

Reaction Setup: Digestion area

5. Digestion	Range	Value
Protease-to-sample Ratio (mass:mass)	user specified	50
Protease Master Reagent Concentration, µg/µL	user specified	0.500
Volume of Protease to Add, µL	5 – 50	9.0
Buffer Concentration for Digest step, M	0.050 – 0.080	0.060
Volume of Digest Reaction, µL	≤ 300	270

Field	Description
Protease-to-Sample Ratio (mass:mass)	The protease-to-substrate protein (sample) ratio. The default value of 50 means the final digest reaction will contain 1 µg of protease for every 50 µg of protein in the sample. Typically, this ratio varies from 1:25 to 1:100. Refer to the recommendations of the protease supplier. Range: Varies Default: 50
Protease Master Reagent Concentration, µg/µL	The concentration of the Protease Master Reagent. This value and the Protease-to-Sample Ratio determine the Volume of Protease to Add. The Protease Master Reagent should be prepared from your protease stock solution. Range: 0.05–1.50 Default: 0.500

Field	Description
Volume of Protease to Add, µL	*Read only*. The volume of Protease to add. The value is calculated using the Protease-to-Sample Ratio and the Protease Master Reagent Concentration. You can compare this value with the Volume of Digest Reaction value, below. Its lower bound is the lowest recommended volume (5 uL), and the upper bound should be no more than 10% of the Volume of Digest Reaction, for best control of reaction pH. Range: 5–50 Default: 9.0
Buffer Concentration for Digest step, Molar	*Read only*. The final concentration of the buffer. The value is calculated using the starting concentration and volumes added from the fields above. Make sure the value remains within the range for optimal for pH control and protease activity. Range: 0.050–0.080 Default: 0.060
Volume of Digest Reaction, µL	*Read only*. The digest reaction volume. The value is calculated using the volumes shown above this field. Make sure the labware you are using can accommodate this volume. The labware should also accommodate any reagents you might want to add to this volume following the overnight digestion. Range: <= 300 Default: 270

Dispensing the solutions

IMPORTANT

To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

Use one of the following methods to set up the reagent plates:

- Automated Reagent Plate Setup
- Manual Reagent Plate Setup

To use Automated Reagent Plate Setup:

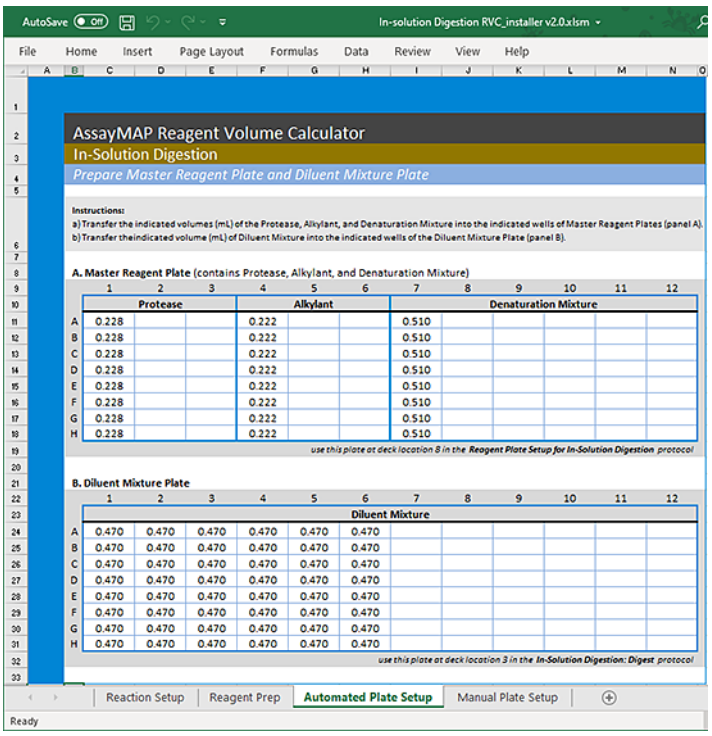
The Master Reagent plate should be prepared at this stage.

1. In the **Reagent Volume Calculator**, display the **Automated Plate Setup** worksheet. See the following figure.
2. Add the designated volumes of Protease, Alkylant, and Denaturation Mixture into the assigned columns of the Master Reagent plate as shown in the **A. Master Reagent Plate** area of the worksheet.
3. Add the volumes of the Diluent Mixture into the assigned columns of the Diluent Mixture plate as shown in the **B. Diluent Mixture Plate** area of the worksheet.

Note: The Diluent Mixture plate is prepared manually because of the large volumes required.

4 To complete the setup, run the Reagent Plate Setup protocol. See "Running the Reagent Plate Setup protocol" on page 255.

Figure Example of Automated Plate Setup worksheet

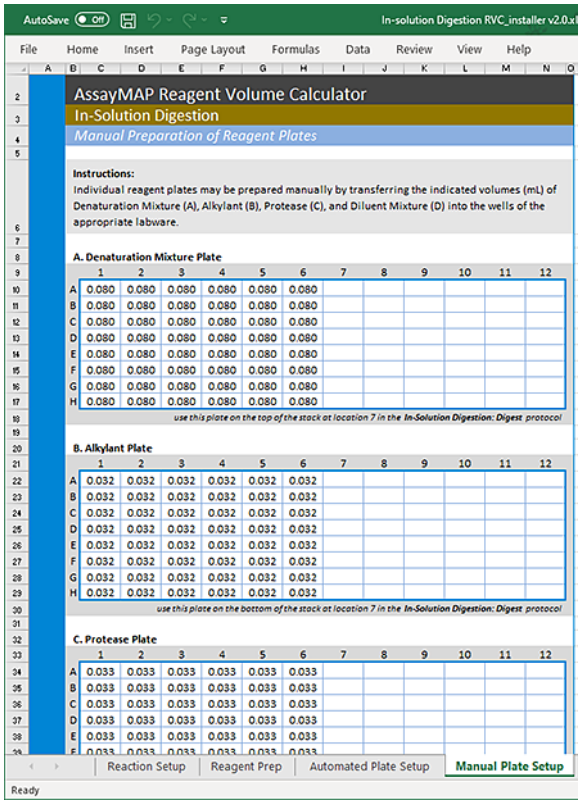


To use Manual Reagent Plate Setup:

1 In the **Reagent Volume Calculator**, display the **Manual Plate Setup** worksheet. See the following figure.

2 Use manual pipettes to prepare reagent plates for the Protease, Alkylant, Denaturation, and Diluent reagents based on their respective plate layouts.

Figure Example of Manual Plate Setup worksheet



Preparing plates for stacking

The In-Solution Digestion: Multi-Plate protocol requires a stack of five 96-well Greiner 650207 U-Bottom plates at deck location 2, regardless of the number of sample plates being used.

IMPORTANT

The automated Bravo plate stacking is required to create a perfectly aligned stack, even if you do not run all the steps or if you have fewer than four sample plates.

To prepare the plates for stacking:

1. Label one plate to be used as a lid for the Protease plate, and label four plates to be used as lids for the sample plates. The four plates that will be used for sample plate lids also function as Syringe Wash Buffer plates.
2. Fill each Syringe Wash Buffer plate with 300 µL per well of buffer, such that the filled columns match the columns of samples in the sample plates.

   You may use manual pipettes for this task, or you may use the Reagent Transfer utility, Reagent Aliquot utility, or the Single Liquid Addition utility.

   Before using a utility, the system must be prepared using the System Startup Utility. For details, see the "System Startup/Shutdown v3.0 User Guide" on page 574.

Preparing the samples

IMPORTANT To minimize evaporation, prepare the samples immediately before run time or keep them covered until you run the protocol.

The In-Solution Digestion application can accommodate a wide range of protein concentrations, which is largely dependent on downstream needs. Viscous samples may require dilution.

When preparing the samples, you must:

- Remove macromolecular particulates that might interfere with accurate pipetting.
- Determine the volume of samples.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris.

Adjusting the sample composition

The concentration of denaturant in the sample should be entered into the In-Solution Digestion Reagent Volume Calculator. See "Using the Reagent Volume Calculator for In-Solution Digestion: Multi-Plate" on page 242.

Determining the volume of sample to digest

The acceptable volume of the sample is highly dependent on the digestion conditions. Use the In-Solution Digestion Reagent Volume Calculator to help determine sample volume. See "Using the Reagent Volume Calculator for In-Solution Digestion: Multi-Plate" on page 242.

Preparing the sample plates

Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 8 to 96 samples per 96 well plate in parallel, and up to four sample plates. The samples should be arranged in contiguous columns, therefore, the samples should be in multiples of 8.

- The volume of samples is limited by the labware you use and the digestion reaction conditions. See "Using the Reagent Volume Calculator for In-Solution Digestion: Multi-Plate" on page 242. The total volume should also include any volume of liquids you intend to add post digestion.

Transferring the samples to the microplate

You can transfer the samples to the microplate that is supplied with the AssayMAP Starter Kit. See "Labware and starter kits" on page 238.

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Single Liquid Addition utility to transfer the samples. For instructions, see one of the following:
   - If you have only one sample plate, see "Reagent Transfer v3.0 User Guide" on page 525.
   - If you have multiple sample plates, see "Single Liquid Addition v2.0 User Guide" on page 542.
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the Reagent Plate Setup protocol

The Reagent Plate Setup protocol uses the AssayMAP Bravo Platform to transfer reagents in the Master Reagent plate to dedicated reagent plates.

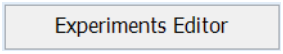
Note: If you have already manually prepared the reagents, you can skip this topic.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you

- If applicable, know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup protocol to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.

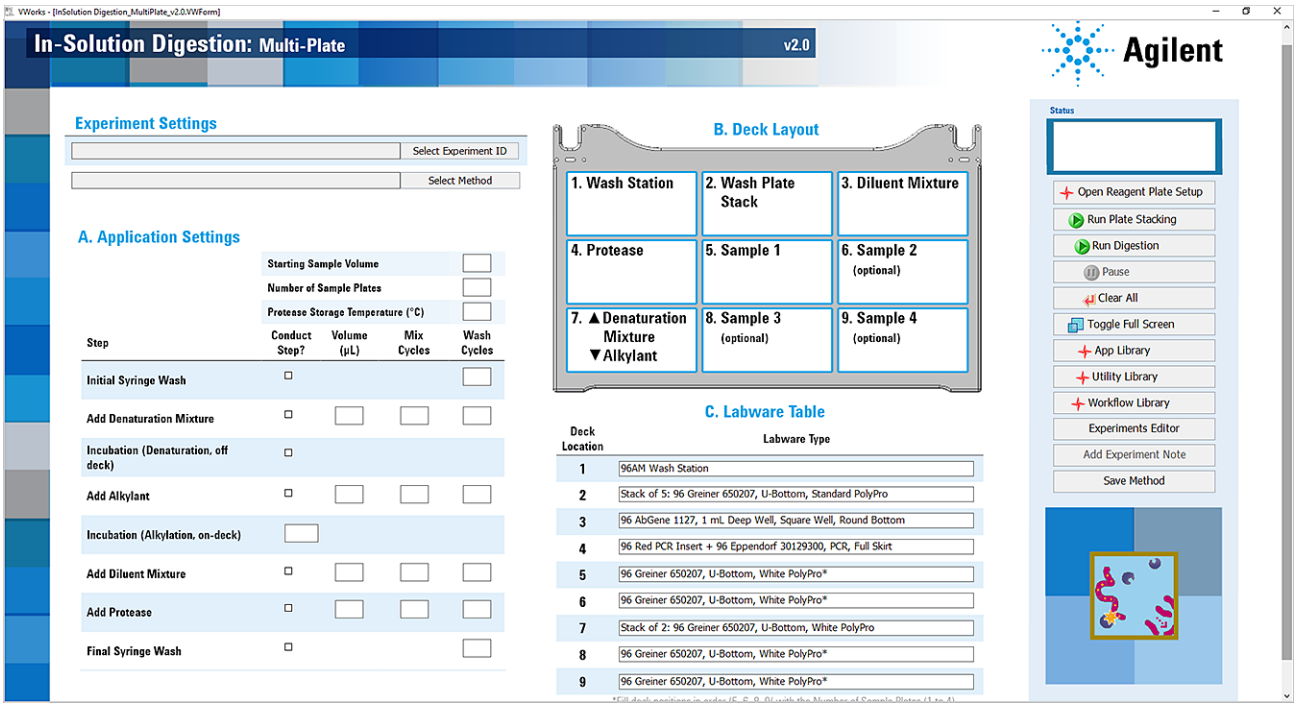
Setting up the Reagent Plate Setup protocol

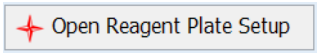
Before starting the protocol, make sure the appropriate selections and values are specified in the application.

To set up the Reagent Plate Setup protocol:

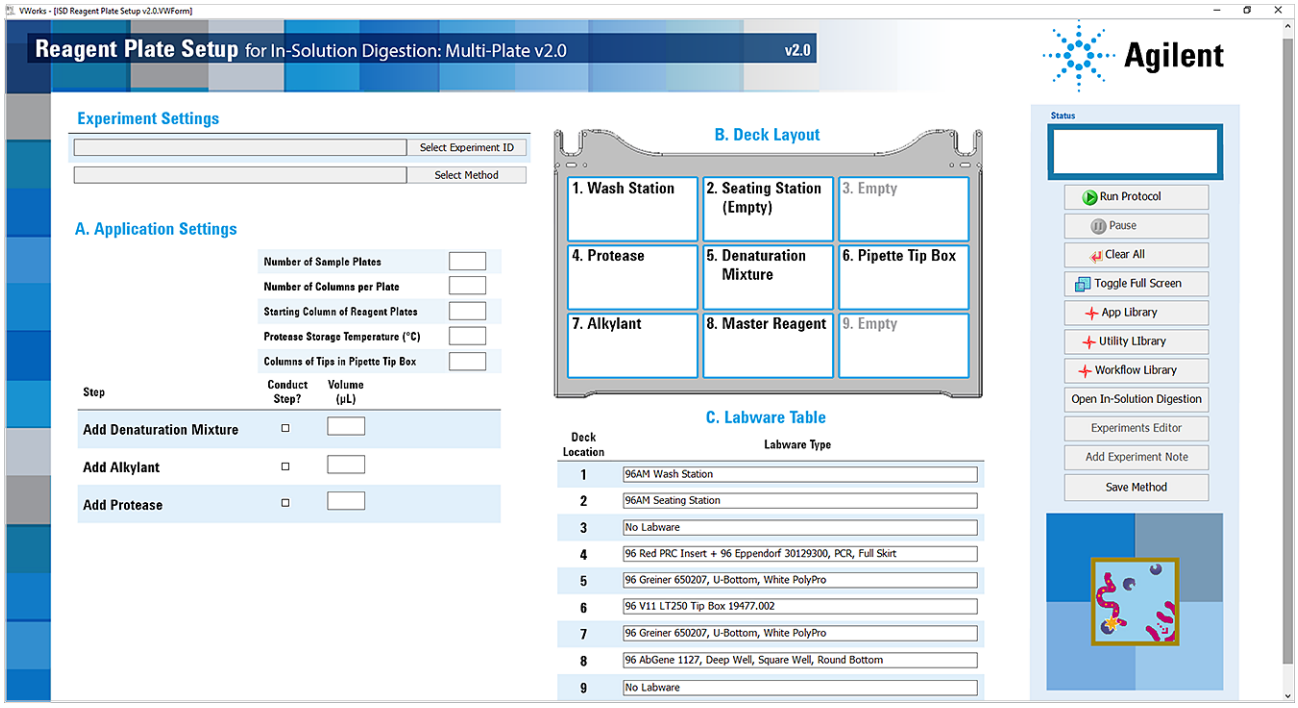
1. Open the **App Library**.
2. Locate **In-Solution Digestion: Multi-Plate**, and then click **App**.

The In-Solution Digestion: Multi-Plate application opens.

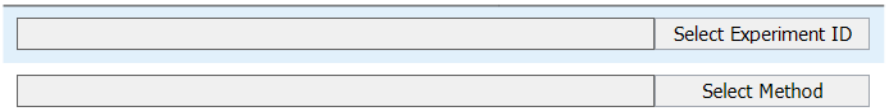


3 In the navigation pane on the right side of the form, click 

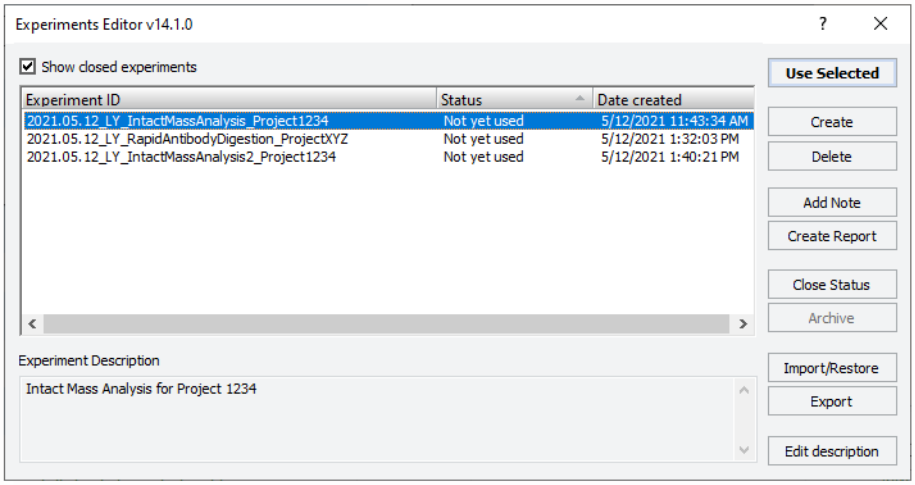
. The Reagent Plate Setup form opens.



4 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



5 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

6 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Running the Reagent Plate Setup protocol" on page 259.
- To modify or create a method, proceed to step 7.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

7 In the **Application Settings** area, select the check boxes of the steps that you want to perform, and enter the values for the selected steps. For details, see "Application Settings for Reagent Plate Setup" on page 258.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experiment ID report is generated.

8 To save the method, click **Save Method**. In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

Application Settings for Reagent Plate Setup

Setting or step	Description	Value	
Number of Sample Plates	Specifies the number of sample plates to be processed.	Default:	1
		Range:	1–4
Number of Columns per Plate	Specifies the number of columns in each reagent plate (Protease, Denaturation, and Alkylant) that will be filled with reagent aliquots. This number must be consistent with the number of columns of samples in each sample plate. The value is used with the Starting Column of Reagent Plates to determine which columns in the reagent plates will receive reagents.	Default:	1
		Range:	1–12
Starting Column of Reagent Plates	Defines the first column in each of the reagent plates that will receive reagent aliquots. This value works with the Number of Columns per Plate to define the range of each reagent plate that will receive reagent.	Default:	1
		Range:	1–12
Protease Storage Temperature	Specifies the temperature set-point that will be used for the Protease plate for the entire Reagent Plate Setup protocol. The temperature controller will not turn off after completion of the Reagent Plate Setup protocol. The assumption is that an In-Solution Digestion run will closely follow the Reagent Setup Run.	Default:	10 °C
		Range:	4–35 °C
Columns of Tips in Pipette Tip Box	Specifies the number of full columns of 250 µL pipette tips in the source tip box. The columns of pipette tips must be contiguous and contain 8 pipette tips per column. If specifying fewer than 12 columns, ensure that no pipette tips are present in the unspecified columns. Make sure that the empty columns are on the right side of the tip box.	Default:	12
		Range:	1–12
Add Denaturation Mixture	Transfers the specified volume, plus the required overage, to the Denaturation Mixture plate.	Default:	30 µL*
		Range:	1–250 µL

Setting or step	Description	Value	
Add Alkylant	Transfers the specified volume, plus the required overage, to the Alkylant plate.	Default:	6 µL*
		Range:	1–250 µL
Add Protease	Transfers the specified volume, plus the required overage, to the Protease plate.	Default:	9 µL*
		Range:	1–250 µL

* Input the value for the volume that you want to add to the samples during the digestion run. The Reagent Volume Calculator automatically adjusts the value to include the recommended overage and provides the volume that you need to add to the plate to transfer the desired volume.

For example, if you set the Add Denaturation Mixture volume to 30 µL, then 50 µL will be added to each active well: 30 µL for the samples and 20 µL for overage.

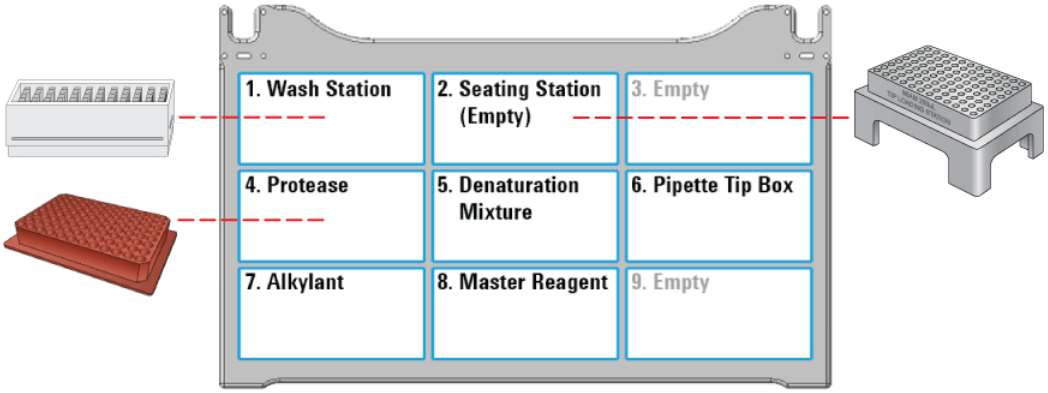
Running the Reagent Plate Setup protocol

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To run the Reagent Plate Setup:

1 Ensure that the reagent plates, pipette tips, and accessories are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

At deck location **4**, ensure that the Red PCR Plate Insert is installed with the **Protease** plate. Otherwise, the protease will not be transferred properly.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Cleaning up after Reagent Plate Setup

When the protocol run is finished, make sure you:

1. Remove the seating station with the used tips (deck location 2), the Master Reagent plate (deck location 8), and the pipette tip box (deck location 6) from the Bravo deck.
2. Discard the excess Master Reagents and used pipette tips following appropriate waste disposal procedures.
3. Keep all remaining labware on the AssayMAP Bravo deck.

IMPORTANT Do not remove the Protease plate from deck location 4, or the stack of reagent plates from deck location 7. These reagents are in their appropriate positions for the In-Solution Digestion: Multi-Plate run.

Running the Digestion protocol

The In-Solution Digestion: Multi-Plate default protocol does the following:

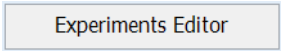
- Denaturant mixture is added to the samples.
- Samples are denatured and reduced at elevated temperature.
- The samples are alkylated.
- The samples are diluted with Dilution mixture.
- Protease is added to the samples.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents, including the plates for stacking. See "Preparing the solutions" on page 241.
- Prepare the samples. See "Preparing the samples" on page 253.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.

- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.

IMPORTANT

Before you run the In-Solution Digestion protocol, you must run the Plate Stacking protocol to stack of five 96-well Greiner 650207 U-Bottom plates at AssayMAP Bravo deck location 2. The Bravo plate stacking is a requirement even if you do not run all the steps or if you have fewer than four sample plates.

Stacking the wash plates and the lid for the protease plate

The In-Solution Digestion: Multi-Plate protocol requires a stack of five 96-well Greiner 650207 U-Bottom plates at deck location 2, regardless of the number of sample plates being used. Ensure that you have prepared the plates for stacking. For details, see "Preparing plates for stacking" on page 252.

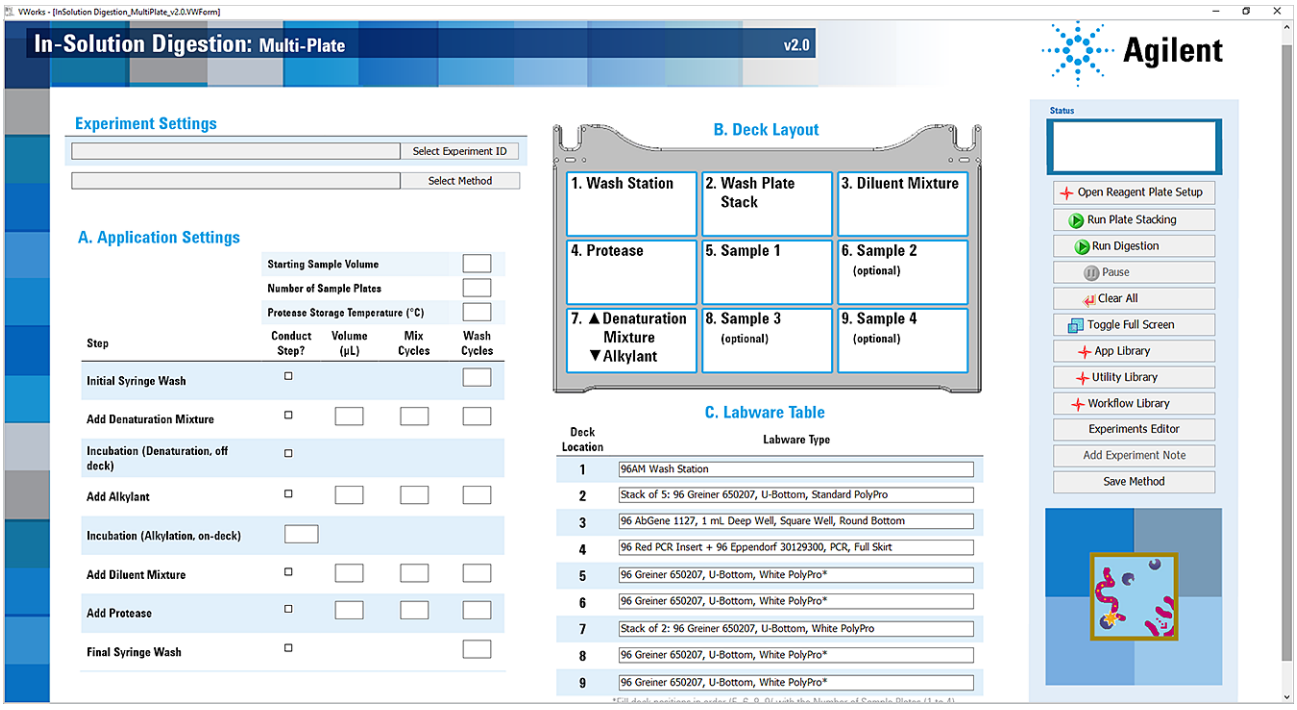
To stack the prepared wash and lid plates:

1 Open the **In-Solution Digestion: Multi-Plate** app:

If the **Reagent Plate Setup for In-Solution Digestion: Multi-Plate** form is already open, click Open In-Solution Digestion in the navigation pane.

Otherwise, locate **In-Solution Digestion: Multi-Plate** in the **App Library**, and then click **App**.

The In-Solution Digestion: Multi-Plate application opens.



2 Click Run Plate Stacking in the navigation pane.

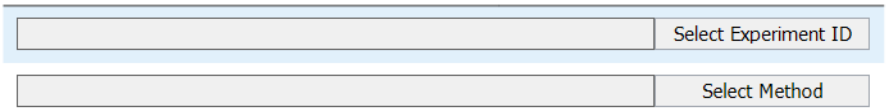
Follow the instructions that appear on the screen after the run starts.

Setting up the Digestion protocol

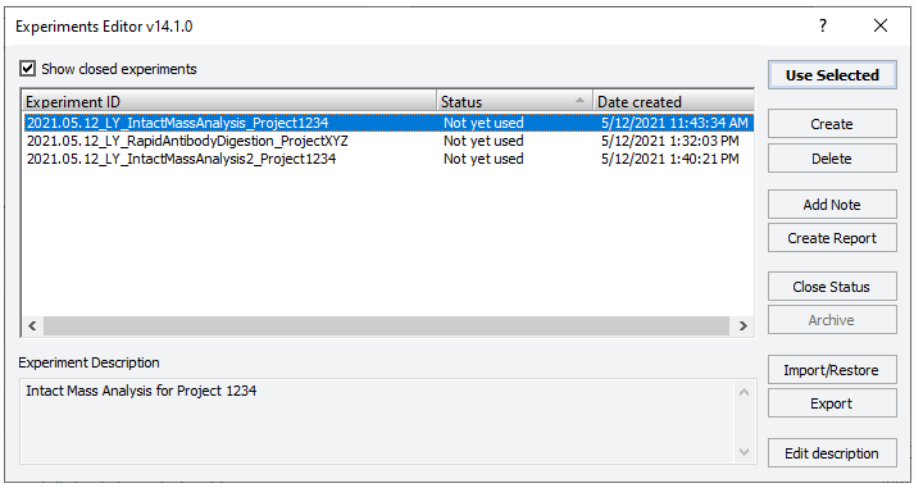
To set up the Digestion protocol:

1 Ensure that the In-Solution Digestion: Multi-Plate form is open.

2 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



3 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

4 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Digestion protocol run" on page 266.
- To modify or create a method, proceed to step 5.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

5 In the **Application Settings** area, do the following:

a Specify the sample information and protease storage temperature. See "Application Settings for In-Solution Digestion protocol" on page 264 for details.

b Select the **Conduct Step?** check box of each step you want to perform, and then specify the settings for the selected steps. For details, see, "Application Settings for In-Solution Digestion protocol" on page 264.

c Click Save Method.

In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

Application Settings for In-Solution Digestion protocol

The following tables give a brief description of each setting. For details, including the full and practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 268.

Table In-Solution Digestion sample and protease settings overview

Setting or Step*	Description	Value	
Starting Sample Volume	The volume of sample in each well in the sample plate.	Default:	15 µL
		Range:	0–300 µL
Number of Sample Plates	The number of sample plates on the AssayMAP Bravo deck.	Default:	1
		Range:	1–4
Protease Storage Temperature	The temperature set-point for the Protease plate (deck location 4) for the duration of the run. The temperature controller will turn off after completion of the In-Solution Digestion run. *Note:* The temperature of the wells will be slightly different than the Peltier set point.	Default:	10 °C
		Range:	4–37 °C

Table In-Solution Digestion Step settings

Setting or Step*	Description		Volume in µL	Mix Cycles	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	Default:	–	–	3
		Range:	–	–	1–10
Add Denaturation Mixture	Aspirates the Denaturation Mixture (deck location 7) into the syringes, and then dispenses it into Sample plate 1 (deck location 5). The solutions in Sample plate 1 are mixed based on the Mix Cycles value, and then the syringes are washed at the wash station (deck location 1) and in the Syringe Wash Buffer plate. This step repeats for each sample plate that is on the deck (locations 6, 8, and 9).	Default:	30	15	3
		Range:	1–250	0–30	0–10
Incubation (Denaturation, off-deck)	Pauses the run after the Add Denaturation Mixture step so that you can manually move the sample plates off deck for incubation, if required, for denaturation and/or reduction. After incubation, you manually place the sample plates back onto the Bravo deck and resume the protocol run.				

Setting or Step*	Description		Volume in µL	Mix Cycles	Wash Cycles
Add Alkylant	Moves the Denaturation Mixture plate from deck location 7 to 3, aspirates the Alkylant (deck location 7) into the syringes, and then dispenses it into Sample plate 1 (deck location 5). The solutions in Sample plate 1 are mixed based on the Mix Cycles value, and then the syringes are washed at the wash station (deck location 1) and in the Syringe Wash Buffer plate. This step repeats for each sample plate that is on the deck (locations 6, 8, and 9).	Default:	6	15	3
		Range:	1–250	0–30	0–10
Incubation (Alkylation, on-deck)	Incubates the sample plates for the specified period (in minutes) on the deck.	Default:	Time: 45 minutes		
		Range:	0–180 minutes		
Add Diluent Mixture	Moves the Denaturation Mixture plate from deck location 3 to 7, aspirates the Diluent Mixture (deck location 3) into the syringes, and then dispenses it into Sample plate 1 (deck location 5). The solutions in Sample plate 1 are mixed based on the Mix Cycles value, and then the syringes are washed at the wash station (deck location 1) and in the Syringe Wash Buffer plate. This step repeats for each sample plate that is on the deck (locations 6, 8, and 9).	Default:	210	15	3
		Range:	1–250	0–30	0–10
Add Protease	Aspirates Protease (deck location 4) into the syringes, and then dispenses it into Sample plate 1 (deck location 5). The solutions in Sample plate 1 are mixed based on the Mix Cycles value, and then the syringes are washed at the wash station (deck location 1) and in the Syringe Wash Buffer plate. This step repeats for each sample plate that is on the deck (locations 6, 8, and 9).	Default:	9	15	3
		Range:	1–250	0–30	0–10
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	Default:	–	–	3
		Range:	–	–	1–10

*For additional protocol guidelines, see "Protocol stepwise guidelines" on page 268.

For a complete list of the robotic movements executed during a run, see "Automation movements during Reagent Plate Setup protocol" on page 269.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

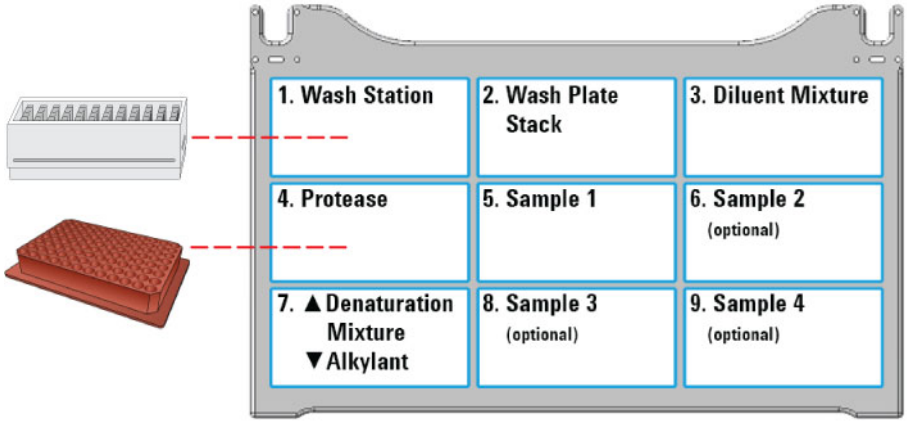
You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can decrease the volumes.

Starting the Digestion protocol run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the Digestion protocol run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.



At deck location 7, ensure the **Denaturation Mixture** plate is stacked atop the **Alkylant** plate.

Note: The Reagent Plate Setup protocol stacks the labware at deck location 7 automatically. If you prepared the reagent plates manually, you must stack the plates manually at deck location 7.

IMPORTANT Do not remove any of the reagent plates from the deck even if the reagent is not being used. Instead, you may use an empty microplate as a place holder. The protocol requires these labware to be in their defined positions to run properly.

The protocol requires five plates for the stack at deck location 2 regardless of the number of sample plates.

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click 

to start the run.

To monitor the progress of the run, check the **Status** box.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

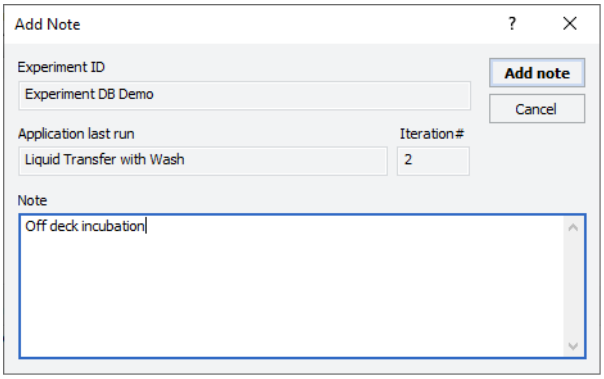
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

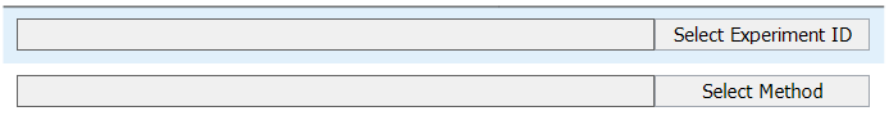
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Digestion protocol to your particular experimental design
- Automation movements during the protocols

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Initial Syringe Wash	The Initial Syringe Wash step removes potential contaminants from the syringes. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. You can increase the number of wash cycles to better clean the syringes. However, be aware that increasing the number of wash cycles also increases protocol run time and reduced syringe lifespan.

Protocol step	Guidelines and notes
Add Denaturation Mixture	The Add Denaturation Mixture step prepares the reaction for denaturation and may include denaturant, reductant, internal standard, and buffer based on user-specified experimental design. The Denaturation Mixture is made during the Reagent Plate Setup protocol as the components are automatically transferred from the Master Plate into the Denaturation Mixture plate.
Incubation (denaturation- off deck)	The denaturation incubation step is used when incubation for denaturation or reduction is required. The step pauses the protocol run so that you can manually move the sample plates off the Bravo deck, manually seal the plates using the PlateLoc Sealer or equivalent device, and incubate the plates for the desired length of time. After the incubation period, remove the seals from the plates, place the plates back on the Bravo deck, and then resume the protocol run.
Add Alkylant	Depending on the specific reagent used for alkylation, the reaction might be light sensitive. To protect the alkylation reaction from light, the Bravo Platform covers the sample plates with wash plates for the duration of the alkylant incubation step.
Incubation (Alkylation on deck)	The Incubation for alkylation step incubates the lidded samples on the Bravo deck. At the end of the incubation period, the wash plates are removed from the sample plates.
Add Diluent Mixture	The Diluent Mixture prepares the reaction for digestion by diluting the denaturant in the sample with buffer to a concentration in which the protease is active and optionally adding a reductant to quench unreacted alkylant.
Add Protease	The In-Solution Digestion protocol is designed to accommodate any proteolytic enzyme. You can add more enzyme using the Single Liquid Addition utility. See "Single Liquid Addition v2.0 User Guide" on page 542.
Final Syringe Wash	The Final Syringe Wash step removes potential contaminants from the syringes. During each Final Syringe Wash cycle, the head aspirates 250 µL of DI water into the syringes using the bare probes, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions.

Automation movements during Reagent Plate Setup protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Reagent Plate Setup protocol.

Protocol step	Head moves to deck location...	Action
Start protocol	NA	Sets the Peltier Thermal Station to the Protease Storage Temperature (°C) specified in the form.
	7	Checks the plate height.
	1	Dispenses any liquid remaining in the syringes. Performs 3 up-and-down cycles of the syringes above the wash station to dry the syringes.
Prepares pipette tips	6	Mounts all the pipette tips present in the tip box. *Note:* The pipette tips are arranged in the tip box in full columns starting at column 1.
	2	Parks the pipette tips in the seating station.
	2	Mounts all the pipette tips in the seating station, except columns 1–3, with a head offset so that the pipette tips in column 4 are mounted onto column 1 (left most column) of the head.
	6	Returns unwanted pipette tips to tip box.
	2	Mounts the pipette tips in column 1–3 of the seating station and moves them to columns 10–12 of the seating station.
Alkylant solution transfer	2	Mounts the pipette tips from seating station column 12 onto column 1 of the head.
	8	Prewets the pipette tips with Alkylant solution.
	8	Aspirates the Alkylant solution, including a small excess.
	7	Dispenses the Alkylant solution.
	8&7	Repeats the preceding two steps as necessary to complete filling the wells.
	8	Dispenses the excess Alkylant solution back into the Alkylant solution.
	2	Ejects the used pipette tips into column 1 of the seating station.

Protocol step	Head moves to deck location...	Action
Protease solution transfer	5	Picks up the Denaturation Mixture Plate.
	7	Puts the Denaturation Mixture Plate on the Alkylant Plate.
	2	Mounts the pipette tips from column 11 of the seating station onto column 1 of the head.
	8	Prewets the pipette tips with Protease solution.
	8	Aspirates the Protease solution, including a small excess.
	4	Dispenses the Protease solution.
	8&4	Repeats the preceding two steps as necessary to complete filling the wells.
	8	Dispenses the excess Protease solution back into the Protease solution.
	2	Ejects the used pipette tips into column 2 of the seating station.
Denaturation Mixture transfer	2	Mounts the pipette tips from column 10 of the seating station onto column 1 of the head.
	8	Prewets the pipette tips with Denaturation Mixture.
	8	Aspirates the Denaturation Mixture, including a small excess.
	7	Dispenses the Denaturation Mixture.
	8&7	Repeats the preceding two steps as necessary to complete filling the wells.
	8	Dispenses the excess Denaturation Mixture back into the Denaturation Mixture.
	2	Ejects the used pipette tips into column 3 of the seating station.

Automation movements during Plate Stacking protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Plate Stacking protocol.

Protocol step	Head moves to deck location...	Action
Start protocol	2	Checks the stack height.

Protocol step	Head moves to deck location...	Action
Stack plates	9	Moves Syringe Wash plate 4 to deck location 2.
	8 6 5 3	Moves Syringe Wash plates 3, 2, 1, and then the Protease Lid to deck location 2.

Automation movements during Digestion protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Digestion protocol. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Start protocol	–	Sets the Peltier Thermal Station to the temperature specified on the form.
	2	Checks the stack height.
	7	Checks the stack height.
	2	Picks up the Protease Lid plate.
	4	Puts the Protease Lid plate on the Protease plate.
Initial Syringe Wash	1	Washes the syringes
Add Denaturation Mixture	7	Aspirates the Denaturation Mixture.
	5	Dispenses the Denaturation Mixture, and then mixes the sample.
	1	Washes the syringes.
	2	Picks up a Syringe Wash plate from the top of the stack.
	5	Puts the Syringe Wash plate on top of the Sample plate, and then performs a Stringent Syringe Wash.
	1	Washes the syringes.
	For each remaining Sample plate: Repeats the Add Denaturation moves starting with aspirating Denaturation Mixture from deck location 7. Returns all Stringent Syringe Wash plates at deck locations 9, 8, 6, and 5 to the plate stack at deck location 2, preserving the plate order so that the same Syringe Wash plates are used for their respective Sample plates.	

Protocol step	Head moves to deck location...	Action
Incubation	Off deck	Protocol pauses and incubation message appears. Operator must manually move Sample plates off deck for incubation, and then move them back on deck and click Continue to resume the run.
Add Alkylant	7	Picks up the Denaturation Mixture plate.
	3	Puts the Denaturation Mixture plate on top of the Diluent plate.
	1	Pauses to wait for temperature to reach set point +/- 5 °C.
	7	Aspirates the Alkylant.
	5	Dispenses the Alkylant and mixes the sample.
	1	Washes the syringes.
	2	Picks up a Syringe Wash plate from the wash plate stack.
	5	Puts the Syringe Wash plate on top of the Sample plate, and then performs a Stringent Syringe Wash.
	1	Washes the syringes.
	For each remaining Sample plate: Repeats the Add Alkylant moves starting with aspirating Alkylant from deck location 7.	
	3	Picks up the Denaturation Mixture plate.
	7	Puts the Denaturation Mixture plate on top of the Alkylant plate.
Add Diluent Mixture	5	Picks up the Syringe Wash plate.
	2	Puts the Syringe Wash plate on top of the stack.
	3	Aspirates the Diluent Mixture.
	5	Dispenses the Diluent Mixture and mixes the sample.
	1	Washes the syringes.
	2	Picks up a Syringe Wash plate from the top of the stack.
	5	Puts the Syringe Wash plate on the Sample plate, and then performs a Stringent Syringe Wash.
	1	Washes the syringes.
	For each remaining Sample plate: Repeats the Add Diluent Mixture moves starting with picking up the Syringe Wash plate.	

Protocol step	Head moves to deck location...	Action
Add Protease	4	Picks up the Protease Lid plate.
	2	Puts the Protease Lid plate on top of the stack. Picks up Protease Lid plate.
	3	Puts the Protease Lid plate on top of the Diluent plate.
	5	Picks up the Syringe Wash plate.
	2	Puts the Syringe Wash plate on top of the stack.
	3	Aspirates the Protease.
	5	Dispenses the Protease and mixes the sample.
	1	Washes the syringes.
	2	Picks up a Syringe Wash plate from the wash plate stack.
	5	Puts the Syringe Wash plate on top of the Sample plate, and then performs a Stringent Syringe Wash.
	1	Washes the syringes.
	For each remaining Sample plate: Repeats the Add Protease moves, except the Protease aspiration happens before picking up of the Syringe Wash plate from the Sample plate and placing it on the stack at deck location 2. Returns all Syringe Wash plates at deck locations 9, 8, 6, and 5 to the plate stack at deck location 2, preserving the plate order.	
	3	Picks up the Protease Lid plate.
	2	Puts the Protease Lid plate on top of the stack.
Final Syringe Wash	1	Washes the syringes.

9 In-Solution Digestion: Single Plate v2.0 User Guide

This chapter contains the following topics:

App description

In-Solution Digestion: Single Plate v2.0. This application enables automated in-solution reactions of 1 to 96 samples in a single 96-well microplate format. The In-Solution Digestion: Single Plate application is designed to maximize simplicity and flexibility for low-to-medium throughput reactions. The procedure consists of five successive liquid-addition steps that each transfer a reagent or mixture of reagents into a common sample plate, followed by incubation on or off deck. The sample volume, incubation duration, incubation temperature, labware, and other parameters can be customized for each of the five addition steps, allowing for almost any manual in-solution reaction method to be automated on the AssayMAP Bravo Platform.

Primary features

The primary features are:

- **Plate lidding**. Enables sample and reagent plates to be introduced with microplate lids to minimize light exposure and evaporation losses.
- **On-deck incubation**. Enables timed sample plate incubation after every reagent-addition step at a user-defined temperature and for a user-defined duration.
- **Mixed-mode pipetting**. For each liquid-addition step, allows the user to choose bare AssayMAP probes or 250-µL pipette tips to transfer the liquid.
- **Method saving**. Enables creating methods for future use. A generic standard in-solution protein digestion method is included that you can use for initial testing purposes or as a template for creating custom digestion processes.
- **Experiment ID database**. Captures the steps executed and the settings used during each run of an application or utility.

Comparison to In-Solution Digestion: Multi-Plate

The following table lists the primary differences between the In-Solution Digestion: Single Plate application and the In-Solution Digestion: Multi-Plate application, which is also included in the Protein Sample Prep Workbench.

Feature	In-Solution Digestion: Single Plate	In-Solution Digestion: Multi-Plate
Throughput	8 to 96 samples per run (1 full plate)	8 to 384 samples per run (up to 4 full plates)
Digestion method	Flexible You can define any digestion method (up to 5 liquid-addition steps per run).	Fixed Specifically designed to run the following: Denature/Reduce > Alkylate > Dilute > Digest.

Feature	In-Solution Digestion: Single Plate	In-Solution Digestion: Multi-Plate
Labware options	Variable You may select labware for sample and reagent plates from a pre-approved list.	Fixed You must use specific labware for every reagent and sample plate.
Pipetting options	For each addition step, you may use pipette tips or the bare probes.	You may use only the bare probes.

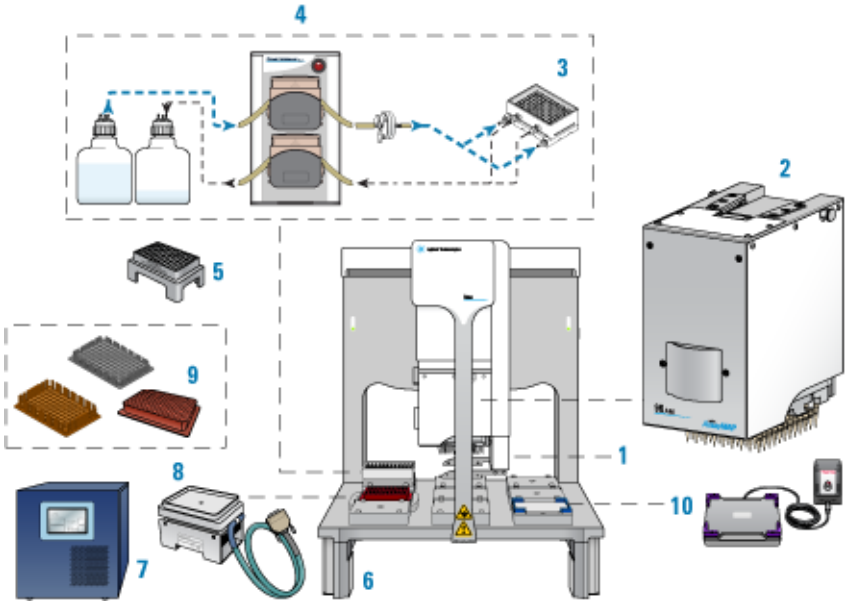
Before you start

This topic lists the required hardware, software, and labware for running the In-Solution Digestion protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which is required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	Peltier Thermal Station with STC controller
8	Custom plate nest
9	Thermal plate insert for efficient heat transfer on the Peltier Thermal Station
10	Orbital Shaking Station with Control Unit

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent
- Heated Incubator

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

Labware

The In-Solution Digestion: Single Plate application does not use AssayMAP cartridges. All pipetting operations are handled in 96-well microplates using either pipette tips mounted on the AssayMAP probes or the bare AssayMAP probes.

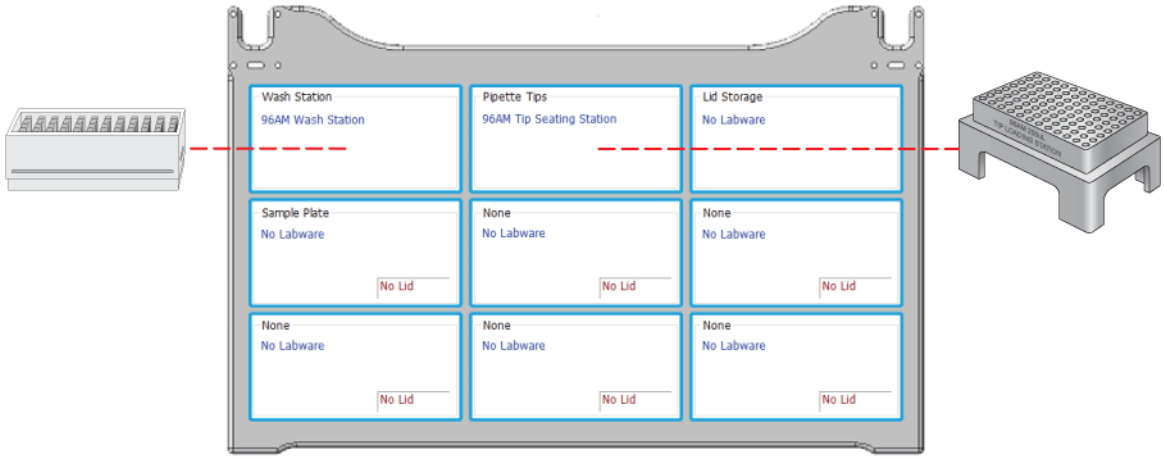
Note: Pipette tips are required for addition steps that include a deep-well plate on the AssayMAP Bravo Platform.

The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
250-µL pipette tips (preloaded in 96AM Cartridge & Tip Seating Station, Agilent G5409-20025)	Agilent 19477-002	2
Lid, Universal**	Agilent 200858-100	4—9
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4—9
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	4—9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4—9
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	4—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	4—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	4—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	4—9

Labware	Manufacturer part number*	Deck location options
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	4—9
96 Eppendorf 96-500_V-bottom, Clear PolyPro	Eppendorf 96/500	4—9
96 Eppendorf 96-1000_U-bottom, Clear PolyPro	Eppendorf 96/1000	4—9
96 Waters 186005837, Clear PolyPro	Waters 186005837	4—9
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	5—9
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	5—9
Reservoir, Seahorse 201254-100, PP, 1 Well, pyramid bottom	Agilent 201254-100	5—9
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	5—9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	5—9
96 Red PCR Insert + Eppendorf 30129300, PCR, Full Skirt	Agilent insert (provided) and Eppendorf 30129300	4
96 Red PCR Insert + Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Agilent insert (provided) and Bio-Rad HSP-9611	4
96 Red PCR Insert + Greiner 652270, PCR, Full Skirt, PolyPro	Agilent insert (provided) and Greiner 652270	4
96 Greiner U-Bottom Thermal Insert + Greiner 650201_U-Bottom, Clear PolyPro	Agilent G5498B#126 and Greiner 650201	4
96 Greiner V-Bottom Thermal Insert + Greiner 651201_V-Bottom, Clear PolyPro	Agilent G5498B#126 and Greiner 651201	4
96 AbGene U-Bottom Thermal Insert + AbGene 1127, Square Well, Round Bottom	Agilent G5498B#127 and ABgene AB-1127	4

*For dimensionally equivalent alternatives and other labware details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**Lidded plates are allowed at these deck locations, not the lid by itself. If you plan to use a lidded plate, ensure that you use only the approved lid type.

Reagents

The types and amounts of reagents required to prepare for the In-Solution Digestion protocol depends on a combination of factors, including specific chemistry requirements, the number of samples to process, and volumes and concentrations of reagents necessary to conduct your digestion process. The Regent Volume Calculator for In-Solution Digestion calculates the reagent volumes required including the overages.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. For more information, see "Preparing the solutions" on page 281.

Preparing the solutions

The In-Solution Digestion: Single Plate application accommodates a wide range of reaction chemistries so that previously optimized conditions can be easily transferred to this automated platform. Agilent recommends that you attempt to transfer your manual in-solution digestion reagent chemistries directly onto the automated platform.

When preparing bulk reagents for use in this application, the total volume required (volume to be pipetted plus overage) for a reagent depends on the labware type. Different types of labware have different well geometries, which results in variability in the amount of excess reagent volume (overage) required to ensure reliable pipetting.

CAUTION

A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for In-Solution Digestion, Single Plate

The Reagent Volume Calculator is a Microsoft Excel file that contains a worksheet. You enter the number of columns to process, the volume for each step in the protocol, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click **Restore Defaults**.

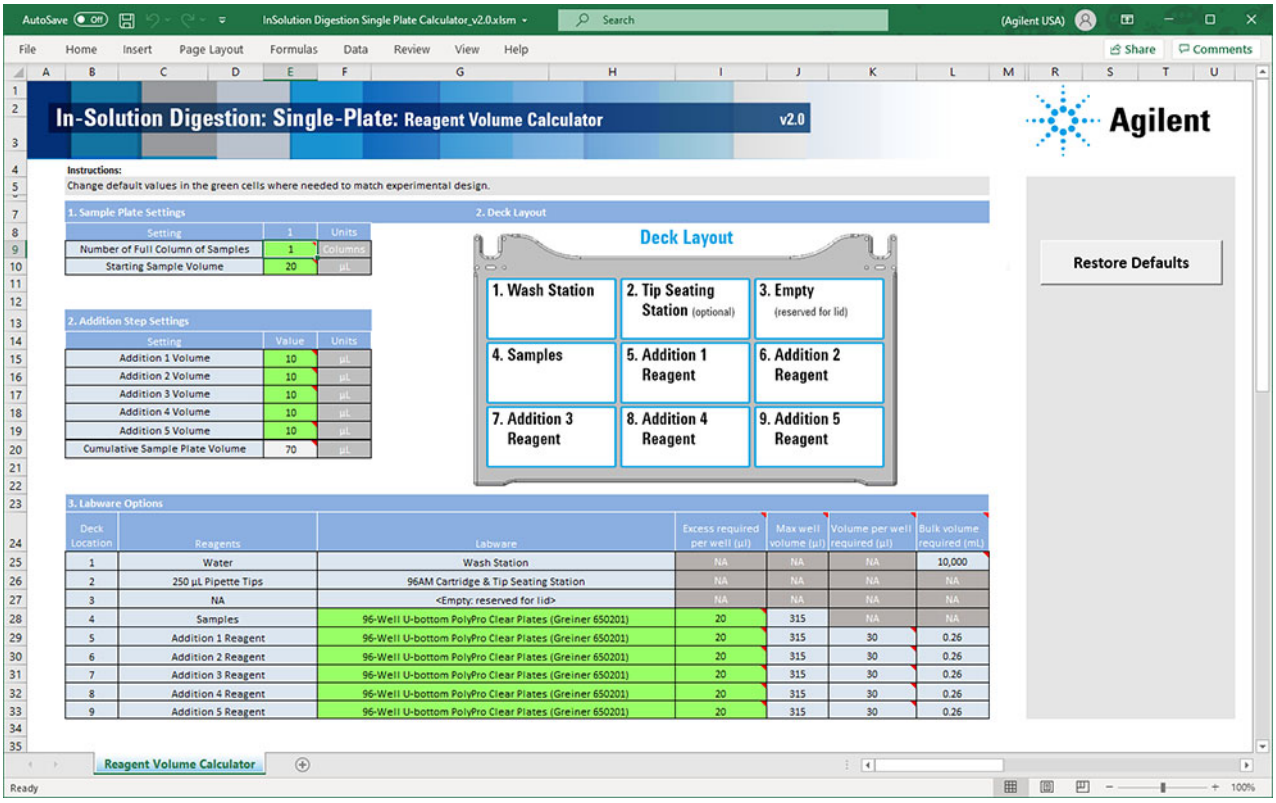
5 Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

To display the corresponding tool tip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figure shows the worksheet of the Reagent Volume Calculator.

Figure Reagent Volume Calculator worksheet



Dispensing the solutions

IMPORTANT

To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

When preparing to do a digestion run, it will often be necessary to aliquot bulk reagents into their individual reagent plates. You can aliquot the reagents using manual pipettes, or use the Reagent Aliquot utility to transfer the reagents automatically. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

IMPORTANT

If you are using fewer than 96 wells, make sure you fill the labware to correspond with the sample layout in the sample plate and the pipette tip positions in the seating station. For more information, see "Preparing the samples" on page 283.

Preparing the samples

IMPORTANT To minimize evaporation, prepare the samples immediately before running the Affinity Purification protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Choose a sample plate with sufficient capacity to hold the total reaction volume, including the sample volume and the volume for all the completed addition steps in the run.
- Transfer the samples to the microplate you want to use for the protocol run.

Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The samples should be arranged in contiguous columns. The position of the samples in the microplate dictates the positions of the buffer solutions in the microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate.

  The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration.

  If using pipette tips for transfer, the AssayMAP Bravo Platform applies differential pressure to seat pipette tips based on the number of full columns of pipette tips. To achieve proper pipette tip seating, entire columns must be used.

Transferring the samples to the microplate

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes or the pipette tips, compromising performance.The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1 Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623

2 If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

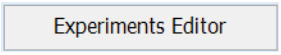
The In-Solution Digestion: Single Plate protocol performs up to five successive liquid-addition steps. Each addition step transfers a reagent or mixture of reagents into a common sample plate, followed by incubation on or off deck. The sample volume, incubation duration, incubation temperature, labware, and other parameters can be customized for each of the five addition steps.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 281.
- Prepare the samples. See "Preparing the samples" on page 283.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See "System Startup/Shutdown v3.0 User Guide" on page 574.
- *Runs that require pipette tips*. Prepare the seating station with the appropriate number and configuration of pipette tips to match the samples to be processed. To prepare the pipette tips, run the Pipette Tip Transfer utility. For details, see "Pipette Tip Transfer v2.0 User Guide" on page 512.

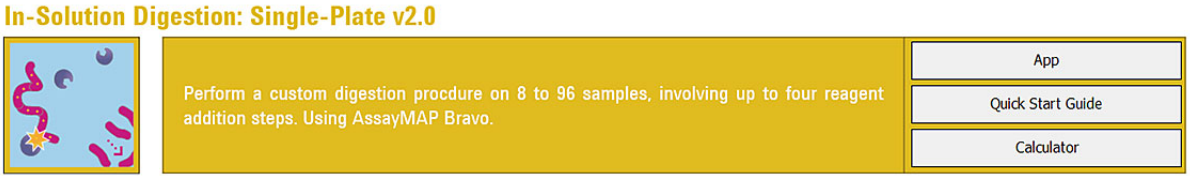
Setting up the protocol

Before you start the protocol, make sure the appropriate selections and values are specified in the In-Solution Digestion application.

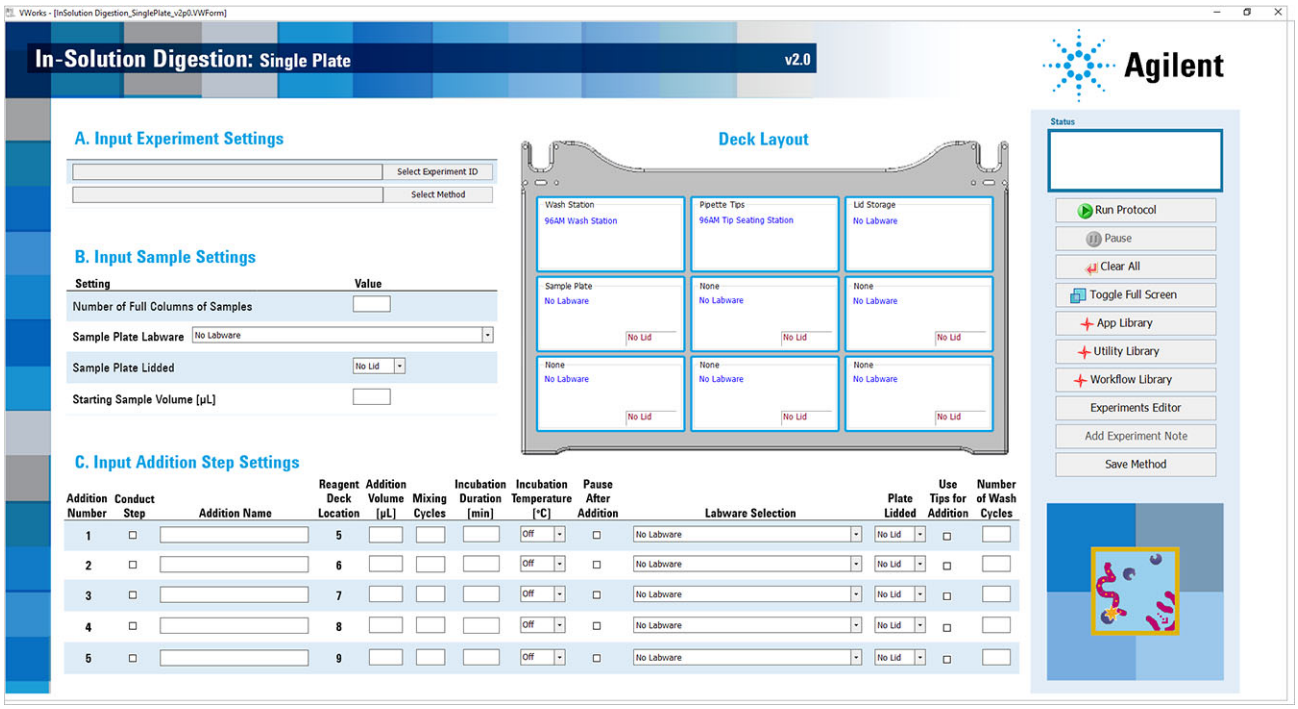
To set up the protocol:

1 Open the **App Library**.

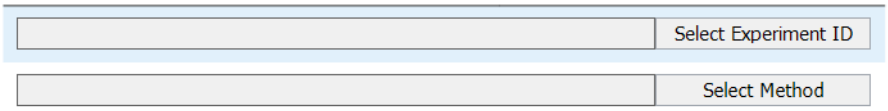
2 Locate **In-Solution Digestion: Single Plate**, and then click **App**.



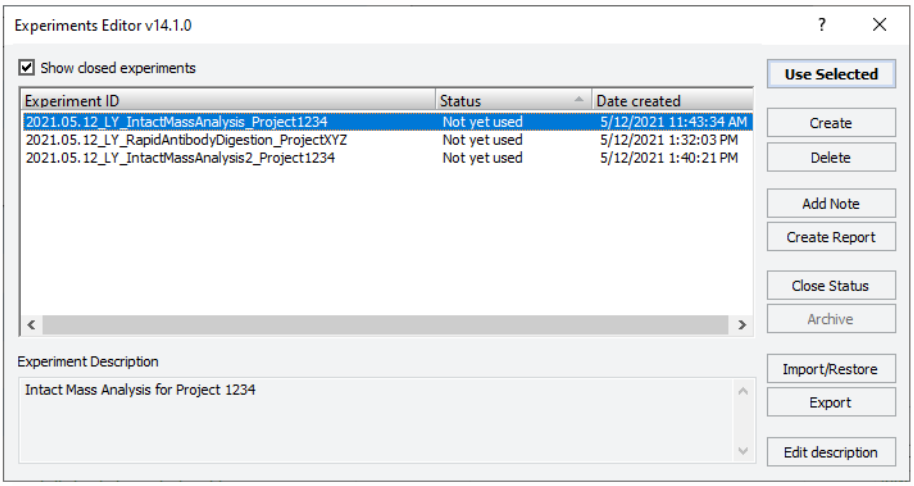
The In-Solution Digestion: Single Plate application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

Note: The software includes an example method file that you can use as a template to create your own methods.

- To run the selected method, go to "Starting the protocol run" on page 290.
- To create or modify a method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 Under **Input Sample Settings**, specify the settings to meet the requirements of your run. For details, see "Input Sample Settings" on page 287.

7 Under **Input Addition Step Settings**, select the check boxes of the steps that you want to perform, and enter the values and labware selections for the selected steps. For details, see "Input Addition Step Settings" on page 288

Note: To avoid potential confusion when a experiment ID report is generated, ensure the following for any unselected addition steps:

- Addition Volume, Mixing Cycles, Incubation Duration, and Number of Wash Cycles boxes are blank.
- Pause After Addition and Use Tips check boxes are cleared.
- Labware Selection is set to No Labware.
- Plate Lidded is set to No Lid.

8 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

A series of error checks are performed. If any errors are detected, a message displays a description of the problem. For details, see "Error messages" on page 299.

Input Sample Settings

The following table gives a brief description of each setting you specify about the Sample Plate at deck location 4.

Table Input Sample Settings overview

Setting	Description	Value range
Number of Full Columns of Samples	Specifies the number of full columns of samples in the sample plate at deck location 4.	1–12
Sample Plate Labware	Specifies the type of labware or labware plus thermal insert that will be placed at deck location 4, which contains the samples to be digested. *Note:* A thermal insert is critical for proper temperature regulation by the Peltier Thermal Station at this location. *Note:* The selected plate must be able to hold the initial volume and the volume that will be added to this plate over the course of the run. *Note:* If a deep-well plate is selected, pipette tips are required for the liquid transfers. For labware options, see "Labware" on page 279.	See "Labware" on page 279.

Setting	Description	Value range
Sample Plate Lidded	Specifies whether the sample plate has a lid. • If you select Lid, ensure that the sample plate has a universal microplate lid present at the start of the run. For details see "Labware" on page 279. • If you select No Lid, ensure the sample plate does not have a lid at the start of the run.	Lid, No Lid
Starting Sample Volume	Specifies the volume of sample that is initially present in the sample plate at the beginning of the run.	0–1000 µL

Input Addition Step Settings

The following table gives a brief description of each setting you specify for each of the five reagent addition steps. For examples of how to vary the addition steps by changing the Incubation and Pause After Addition settings, see "Assay development guidelines and protocol notes" on page 292.

The values that you use can vary dramatically from experiment to experiment. For an example of an approach that uses values like those in the supplied method, Standard Digestion 1.mth, see Agilent Application Note 5991-4872EN in the "Reference library" on page 308.

Table Input Addition Step Settings overview

Setting	Description	Value range
Conduct Step	Indicates whether to perform the corresponding addition step.	Selected, Not selected
Addition Name	Specifies a name for the addition step.	–
Addition Volume	Specifies the volume of reagent that will be transferred from the reagent plate into the sample plate at deck location 4. *Note:* The Reagent Deck Location column shows the deck location of the reagent plate. *Note:* Accuracy and precision decrease with volumes less than 5 µL.	0–1000 µL
Mixing Cycles	Specifies the number of mixing cycles that will be used to mix the samples and reagents after the reagent addition. *Note:* The number of mixes must be determined empirically. In general, if the total volume in the wells are greater than 200 µL or the viscosity is high, the mix cycles must be greater than 10.	0–100
Incubation Duration	Specifies the amount of time that the sample plate will be incubated (deck location 4) after the reagent is added and the mixing is completed. The reagent addition step will not start until the reading on the Peltier Thermal Station is within 5 °C of the specified incubation temp. *Note:* The combination of setting the Incubation Duration to 0 and selecting Pause After Addition allows off-deck incubation for as long as desired. This may be required for high-temperature incubations where evaporation or condensation on the plate lid is a concern.	0–1000 min

Setting	Description	Value range
Incubation Temperature	Specifies the temperature set point of the Peltier Thermal Station during the sample plate incubation (deck location 4).	OFF, 4–110 °C
Pause After Addition	Pauses the protocol after completing the tasks in the step and before starting the next addition step. See "Assay development guidelines and protocol notes" on page 292 for instructions on effectively using the Pause feature.	Selected, Not selected
Labware Selection	Specifies the labware type that will be present at the corresponding Reagent Deck Location for the step.	See "Labware" on page 279.
Plate Lidded	Specifies whether the reagent plate (deck locations 5–9) will have a lid during the run. • If you select Lid, ensure that the reagent plate associated with the step has a universal microplate lid present at the start of the run. For details see "Labware" on page 279. • If you select No Lid, ensure the reagent plate associated with the step does not have a lid.	No Lid, Lid
Use Tips for Addition	Specifies whether pipette tips (deck location 2) will be used instead of the bare probes to transfer this reagent into the sample plate. • To use bare probes, clear the check box. • To use pipette tips, select the check box. You must fill the seating station with the appropriate number and configuration of pipette tips to match the samples to be processed. To prepare the pipette tips, run the Pipette Tip Transfer utility ("Pipette Tip Transfer v2.0 User Guide" on page 512).	Selected, Not selected
Number of Wash Cycles	Specifies the number of wash cycles to be performed at the wash station after the reagent addition step has completed. • If the Use Tips for Addition check box is selected, this step washes the pipette tips. • If the Use Tips for Addition check box is cleared, the protocol washes the bare syringe probes.	0 - 10

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can decrease the volumes.

If your In-Solution Digestion protocol requires pipette tips, you must use pipette tips for the mock run. For instructions on how to set up the pipette tips in the seating station, see "Pipette Tip Transfer v2.0 User Guide" on page 512.

Starting the protocol run

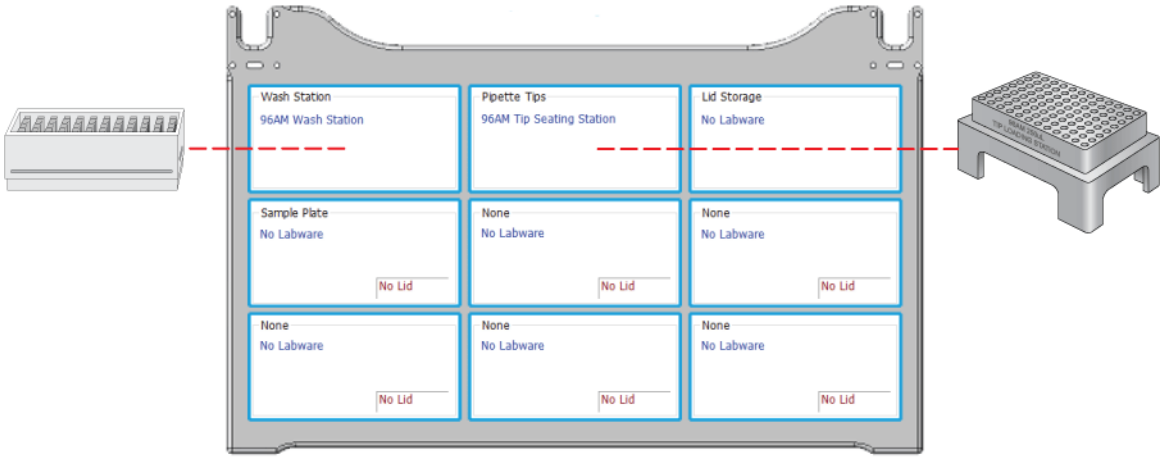
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

- Place the filled Sample Plate at deck location 4 and the filled reagent plates at their respective deck locations.
- Ensure that all plates that require lids have been properly lidded, and no lids are present on other labware.
- If pipette tips are required, ensure the pipette tips are in the seating station at deck location 2.

Make sure the labware are properly seated on the Bravo deck.

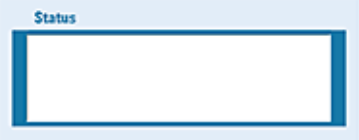


IMPORTANT If lids are specified in the method, ensure that you use the universal microplate lid. See "Labware" on page 279. Ensure that the lids are on the corresponding plates.

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

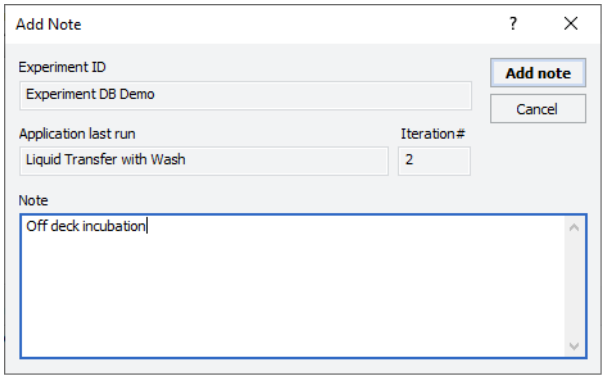
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

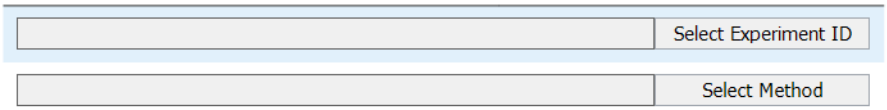
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click  to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Guidelines and examples on how to vary the protocol settings
- Automation movements during an example protocol run

For a description of the settings, see "Input Sample Settings" on page 287 and "Input Addition Step Settings" on page 288.

Examples of how to vary Addition steps by changing Incubation and Pause settings

The following examples demonstrate different ways of defining the following settings to produce different AssayMAP Bravo behaviors:

- Incubation Duration (min)
- Incubation Temperature (°C)
- Pause After Addition

Note: The choice to use a lid or not is experiment dependent. Agilent recommends using a lid when doing incubations at elevated temperatures if evaporation is a concern.

Example 1. Basic addition of a reagent to the samples

This example shows the settings for a simple addition step that uses the bare AssayMAP probes to transfer 10 µL from the Reagent plate at deck location 5 into the Sample plate at deck location 4.

The settings are as follows:

- Incubation Duration 0 min
- Incubation Temperature OFF
- Pause After Addition not selected

Figure Example 1 settings

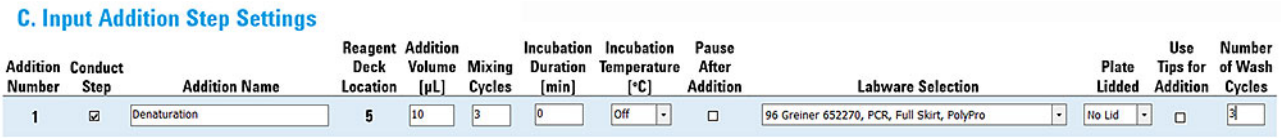


Table Example 1: AssayMAP Bravo actions during the protocol

Action		Deck location
a	Uses the bare probes to aspirate 10 µL from the Reagent plate into the syringes.	5
b	Dispenses the 10 µL into the Sample plate.	4
c	Performs three mix cycles in the Sample plate to mix the contents.	4
d	Performs three syringe washes at the wash station.	1
e	Continues on to Addition Steps 2 to 5.	varies

When to use these settings: In general, when incubation and deck manipulation are not required, set these addition steps as follows:

- Incubation Duration (min) to 0
- Incubation Temperature (°C) to OFF

However, if an Addition step with no incubation follows a step that includes incubation, make sure that you account for the ramp time to change the temperature. For details, see "Additional incubation control considerations" on page 297.

Example 2: Addition with on-deck incubation

This example uses the Peltier Thermal Station to automate an incubation step. The addition step settings include on-deck incubation after the liquid transfer to the Sample plate.

The settings are as follows:

- Incubation Duration 45 min
- Incubation Temperature 37 °C
- Pause After Addition not selected

Figure Example 2 settings

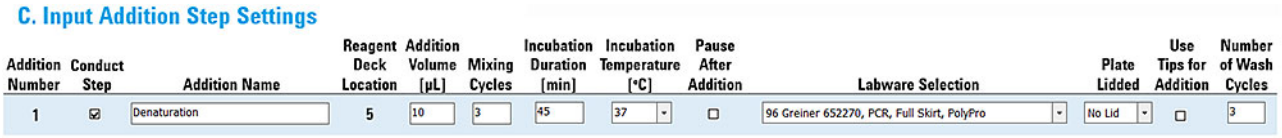


Table Example 2: AssayMAP Bravo actions during the protocol

Action		Deck location
a	Sets the Peltier Thermal Station to 37 °C. *Note:* The heater is activated before the liquid transfer because it takes about 5 minutes for the temperature to reach the set point.	4
b	Uses the bare probes to aspirate 10 µL from the Reagent plate into the syringes.	5
c	Dispenses the 10 µL into the Sample plate.	4
d	Performs three mix cycles in the Sample plate to mix the contents.	4
e	Starts the incubation timer when the Peltier Thermal Station reaches 37 °C.	–
f	Performs three syringe washes at the wash station.	1
g	Remains idle for the remainder of the 45-minute incubation time.	–
h	Continues on to Addition Steps 2 to 5.	varies

When to use these settings: Set the Incubation Duration and Incubation Temperature values for all addition steps where the Sample plate incubation will be conducted automatically on the AssayMAP Bravo deck.

Example 3: Addition with a pause

This example shows settings for an addition step that includes a pause after the liquid transfer to the Sample plate.

The settings are as follows:

- Incubation Duration 0 min
- Incubation Temperature OFF
- Pause After Addition selected

Figure Example 3 settings

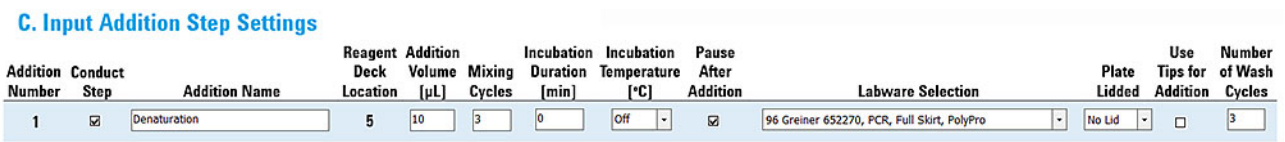


Table Example 3: AssayMAP Bravo actions during the protocol

Action		Deck location
a	Uses the bare probes to aspirate 10 µL from the Reagent plate into the syringes.	5
b	Dispenses the 10 µL into the Sample plate.	4
c	Performs three mix cycles in the Sample plate to mix the contents.	4
d	Performs three syringe washes at the wash station.	1
e	A Pause message appears on the screen and the AssayMAP Bravo Platform stops executing new tasks. The action remains paused until you click the Continue button that appears on the screen.	–
f	Continues on to Addition Steps 2 to 5.	varies

When to use these settings: Select the Pause After Addition option to pause the run so that you can do the following:

- Replace used pipette tips with new pipette tips.
- Remove the Sample plate from the deck for off-deck incubation.

Example 4: Addition with on-deck incubation and a pause

This example uses the Peltier Thermal Station to automate an incubation step. After the incubation, the run will pause to allow the operator to modify something on the deck.

This settings are as follows:

- Incubation Duration 60 min
- Incubation Temperature 60 °C
- Pause After Addition check box selected

Figure Example 4 settings

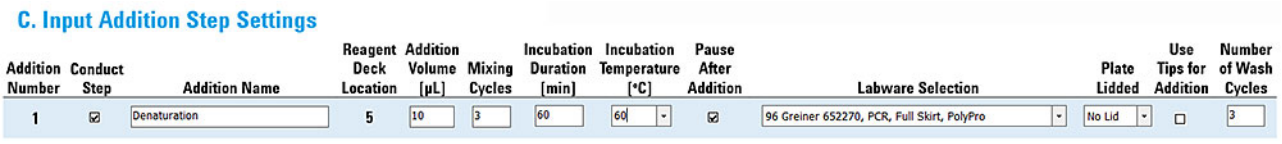


Table Example 4: AssayMAP Bravo actions during the protocol

Action		Deck location
a	Sets the Peltier Thermal Station to 60 °C. *Note:* The heater is activated before the liquid transfer because it takes about 5 minutes for the temperature to reach the set point.	4

Action		Deck location
b	Uses the bare probes to aspirate 10 µL from the Reagent plate into the syringes.	5
c	Dispenses the 10 µL into the Sample plate.	4
d	Performs three mix cycles in the Sample plate to mix the contents.	4
e	Starts the incubation timer when the Peltier Thermal Station reaches 60 °C.	–
f	Performs three syringe washes at the wash station.	1
g	Remains idle for the remainder of the 60-minute incubation time.	–
h	A Pause message appears on the screen and the AssayMAP Bravo Platform stops executing new tasks. The action remains paused until you click the Continue button that appears on the screen. *Note:* The Peltier Thermal Station remains at temperature (60 °C in this case) until the Pause message is cleared.	–
i	Continues on to Addition Steps 2 to 5.	varies

When to use these settings: Use these settings to incubate a plate.

Example 5: Addition with only Incubation Temperature Set and Pause Activated

This example uses the Peltier Thermal Station to partially automate an incubation step. The on-deck incubation of the Sample plate is activated by setting the Incubation Temperature, but no Incubation Duration is specified. The Pause After Addition option is selected, which will cause the incubator to be active during the pause so that the operator can control the duration of the incubation.

The settings are as follows:

- Incubation Duration 0 min
- Incubation Temperature 60 °C
- Pause After Addition selected

Figure Example 5 settings

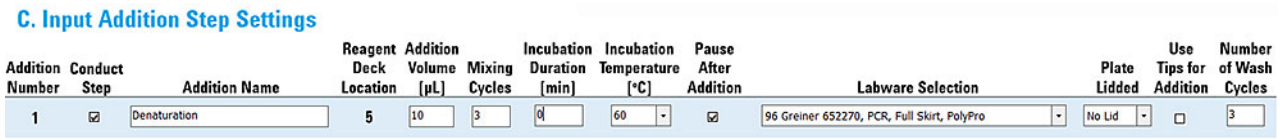


Table Example 5: AssayMAP Bravo actions during the protocol

Action		Deck location
a	Sets the Peltier Thermal Station to 60 °C. *Note:* The heater is activated before the liquid transfer because it takes about 5 minutes for the temperature to reach the set point.	4
b	Uses the bare probes to aspirate 10 µL from the Reagent plate into the syringes.	5
c	Dispenses the 10 µL into the Sample plate.	4
d	Performs three mix cycles in the Sample plate to mix the contents.	4
e	Performs three syringe washes at the wash station.	1
f	A Pause message appears on the screen and the AssayMAP Bravo Platform stops executing new tasks. The action remains paused until you click the Continue button that appears on the screen. *Note:* The Peltier Thermal Station remains at temperature (60 °C in this case) until the Pause message is cleared.	–
g	Continues on to Addition Steps 2 to 5.	varies

When to use these settings: Use this combination of settings when it is necessary to apply a robust seal to the plate, but still use the on-deck incubator to heat or cool the samples.

Note: In this case, the operator is responsible for timing the incubation step because the system does not keep track of the time that the plate was removed or returned to the deck.

Additional incubation control considerations

IMPORTANT

The Peltier Thermal Station requires time to reach the temperature set point. Therefore, the device must be activated before incubation at the set temperature begins.

The time that it takes the Peltier Thermal Station to reach the set temperature is called *ramp* time. The temperature is in flux during the ramp time. The ramp time duration greatly depends on whether the Peltier Thermal Station is being used to actively control the temperature to the next set point or not. The following table provides approximate temperature ramp times for common incubation scenarios.

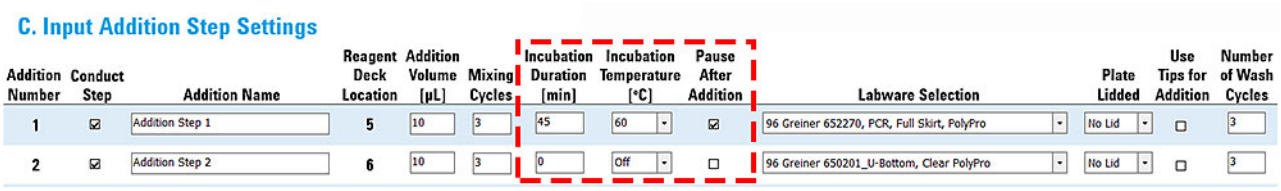
Table Examples of approximate ramp time (min) to reach target temperature

Scenario	Uses Peltier Thermal Station?	Start temperature (°C)	Target temperature (°C)	Ramp time (min), approximate
1	Yes	25	60	< 5
2	Yes	25	10	< 5
3	Yes	60	10	< 10
4	Yes	37	25	< 5
5	No	37	25	> 15
6	No	60	25	> 30
7	No	10	25	> 20

The following examples illustrate why temperature ramp times are an important consideration when defining an In-Solution Digestion method.

Example 1: Addition with incubation followed by addition with heater turned off

Figure Example 1 settings



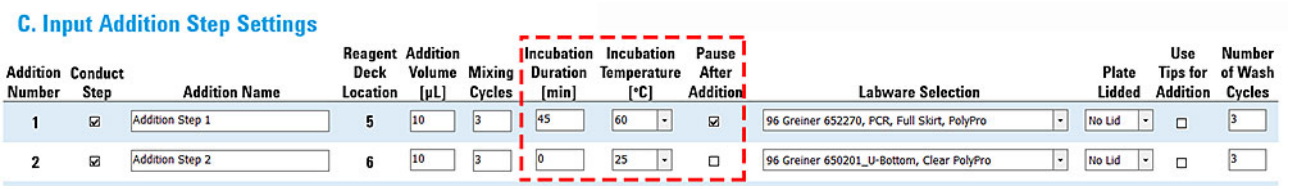
In this example:

- Addition 1 requires an incubation of 45 minutes at 60 °C.
- Addition 2 does not require incubation, so the Incubation Temperature is set to Off.

Intuitively, it makes sense to turn off the incubator if it is not required, but this could have unintended consequences for Addition 2 and subsequent additions. After completing Addition 1, the Peltier Thermal Station will be turned off in preparation for Addition 2, but its temperature will still be very close to 60 °C. According to the preceding table of example ramp times, the Peltier Thermal Station requires at least 30 minutes to return to ambient temperature (~25 °C). The elevated temperature at deck location 4, where the Peltier Thermal Station is installed, could be problematic if later addition steps are sensitive to elevated temperature. In most cases, this problem can be mitigated by using the Peltier Thermal Station to actively cool the temperature back to ambient temperature, as described in Example 2.

Example 2: Addition with incubation followed by addition with heater at ambient temperature

Figure Example 2 settings



In this example:

- Addition 1 requires an incubation of 45 minutes at 60 °C.
- Addition 2 requires at 25 °C (ambient temperature) at deck location 4.

These settings will cause the Peltier Thermal Station to actively cool the temperature from Addition 1 to ambient temperature for Addition 2. The approximate time required is 5 minutes for this temperature change. See the preceding table of example ramp times.

Error messages

Before executing an In-Solution Digestion: Single Plate method, the application performs several error checks to ensure that a feasible run has been specified.

The following table lists some of the types of error checks.

Error check	Description
Sample Plate Capacity	Compares the Initial Sample Volume input to the Sample Plate Labware selection to verify that it does not exceed the labware volume capacity.
Reagent Plate Capacity	Compares each Reagent Addition Volume input to its respective Labware Selection to ensure that it does not exceed the labware volume capacity.
Cumulative Volume	Calculates the cumulative digest volume (Initial Sample Volume + sum of Reagent Addition Volumes) to ensure that the final digest volume does not exceed the Sample Plate Labware volume capacity.
Sample Plate Pipette Tip Compatibility	If a deep-well plate type is selected for the Sample Plate Labware, each addition step is verified to ensure that the Use Pipette Tips for Addition? option is selected.
Reagent Plate Pipette Tip Compatibility	If a deep-well plate type is selected for any of the five reagent Labware Selections, the corresponding Use Pipette Tips for Addition? setting is verified to ensure that pipettes will be used for the transfer.
No Addition Steps	Checks to see that at least one reagent addition step has been specified by ensuring that not all of the Reagent Names settings have been set to NONE.

If a problem is identified, an error message displays. The following table lists some of the error messages, reasons for the error, and a description of how to correct the error.

Method Status error message	Reason for error	How to correct the error
File chosen is not a valid Method file.	The method file selected is for a different application.	Click **Select Method**, and then select a valid method file.
File type is incorrect. Please choose a correct Method file.	The file selected is not a method file.	Click **Select Method**, and then select a valid method file.
Error: Total volume will exceed Sample Labware capacity (0 µL). Please select a larger capacity Sample Labware.	No labware is selected for the Sample Plate.	Under **Input Sample Settings**, select the labware that you are using from the **Sample Plate Labware** list.
Error: Total volume will exceed Sample Labware capacity (n µL). Please select a larger capacity Sample Labware.	The initial sample volume exceeds the capacity of the selected labware type.	Do one of the following: • Select a labware type with a larger capacity from the **Sample Plate Labware** list. • Reduce the value in the **Starting Sample Volume** box to a number that is within the capacity of the current labware selection. For a list of maximum volumes by labware type, see "Labware" on page 279.
Error: Sample Plate is a type of deep well labware. Tips are needed in all steps.	Some of the addition steps specify the use of the AssayMAP bare probes, which cannot access the bottom of most deep-well plates. Pipette tips are required for addition steps that include a deep-well plate on the AssayMAP Bravo Platform.	Do one of the following: • Select the **Use Tips for Addition** check box for each Addition step to ensure that pipette tips are used for the transfers instead of the AssayMAP probes. • If you do not want to use pipette tips for the transfers, ensure that no deep-well plates are selected in the **Sample Plate Labware** list and the **Labware Selection** lists for the Addition steps.
Error: Check Step 1 for volume/labware/ tip combination.	The Volume input for one or more of the Addition Steps is greater than the volume capacity for the selected labware type.	Do one of the following for the corresponding Addition step: • Select a labware type with a larger capacity from the **Labware Selection** list. • Reduce the value in the **Addition Volume** box to a number that is within the capacity of the current labware selection. For a list of maximum volumes by labware type, see "Labware" on page 279.

Method Status error message	Reason for error	How to correct the error
Error: Check Step 1 for volume/labware/ tip combination. Error: Total volume will exceed Sample Labware capacity. Please select a larger capacity Sample Labware.	The cumulative volume in the sample plate, after all addition steps have completed, would be greater than the capacity of the selected Sample plate labware type.	Do one of the following: • Select a labware type with a larger capacity from the **Sample Plate Labware** list. • Reduce the values in the **Starting Sample Volume** box and/or the **Addition Volume** boxes to volumes that are within the capacity of the current labware selection. For a list of maximum volumes by labware type, see "Labware" on page 279.
There are no reagent steps included on the form. Please double check and start the protocol again.	The Addition Name for all five addition steps is set to None, which causes all steps to be skipped.	For each addition step you want to include, select or type a name in the **Addition Name** box. Ensure that you have entered settings for at least one addition step in the **Input Addition Step Settings** area.
Error: Incubation temperature for Addition step 1 is out of range.	The Incubation Temperature (°C) is set to a value that is outside of the allowable range (4 to 100 °C).	If you want to specify an on-deck incubation, enter a value within the allowable range (4 to 100 °C) in the **Incubation Temperature (°C)** box. If you do not want to specify a temperature, select **OFF**. **IMPORTANT** For guidelines on how to control the temperature changes between addition steps, see "Additional incubation control considerations" on page 297.
Error: No labware associated with Addition step 1.	The Labware Selection is set to No Labware for one or more addition steps.	For each addition step you want to include, select the corresponding labware from the **Labware Selection** list.
Error Check Step 1 for volume/labware/tip combination.	A deep-well plate is selected for one or more of the addition steps, but these steps are specified to use AssayMAP Probes, which cannot access the bottom of most deep-well plates.	Do one of the following: • Select the **Use Tips for Addition** check box for each Addition step to ensure that pipette tips are used for the transfers instead of the AssayMAP probes. • If you do not want to use pipette tips for the transfers, ensure that no deep-well plates are selected in the **Labware Selection** lists for the Addition steps.
Error: Addition step 1 labware is a reservoir, lids cannot be used.	The selected labware type cannot have a lid, but the Plate Lidded setting is set to Lid.	Do one of the following: • Select **No Lid** in the **Plate Lidded** box for this Addition step. • Select a type of labware that can use a lid in the **Labware Selection** list for this Addition.

Automation movements during the protocol

In-Solution Digestion: Single Plate is designed to mimic common manual in-solution digestion procedures for successively adding digestion reagents to samples and then incubating. In this application, a 96-well sample plate is placed at Bravo deck location 4, and up to five 96-well plates containing digestion reagents are placed at deck locations: 5—9. During the protocol, the AssayMAP Bravo Platform transfers each of the digestion reagents into the sample plate, one at a time, with optional heated incubation steps after each reagent addition. The automation movements are dependent on the choices selected in the method.

The following figure shows an example of an In-Solution Digestion method and the following table lists the automation movements for this example.

Figure Example method settings

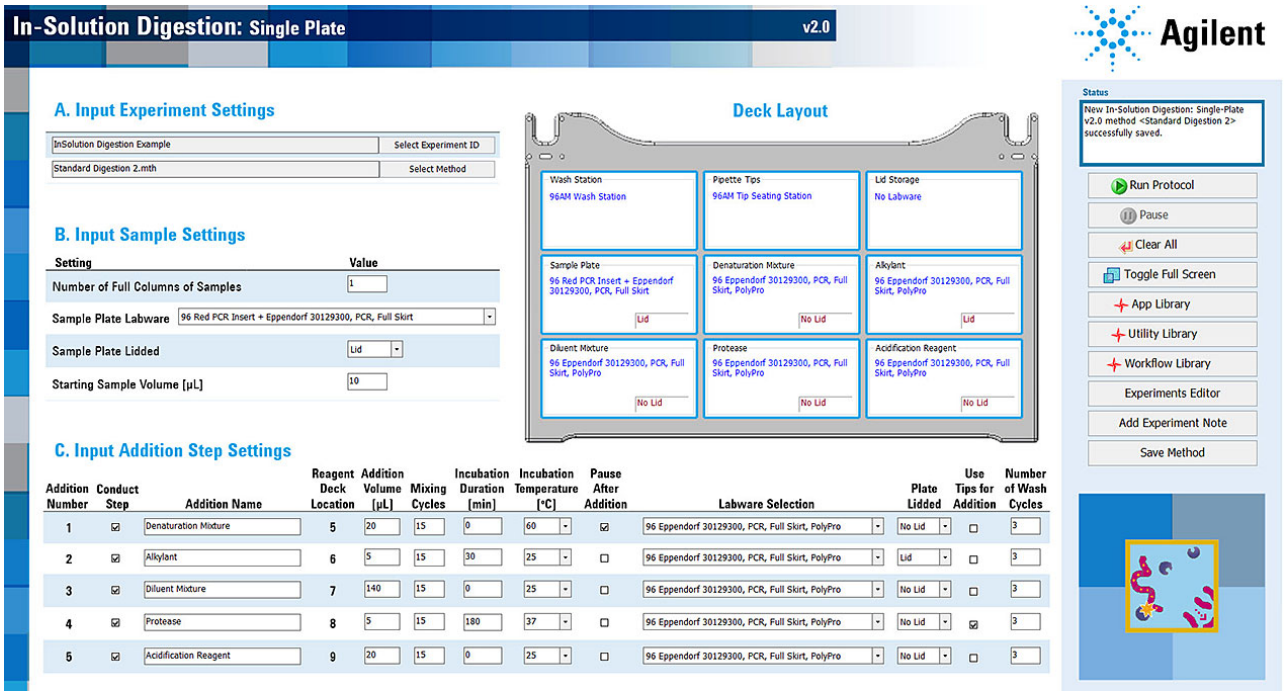


Table Automation movements for example method

Protocol step	Head moves to deck location...	Action
Starting protocol	2	Parks all cartridges that might have been loaded on the head from a previously aborted protocol.
	1	Dispenses any liquid remaining in the syringes into the wash station.

Protocol step	Head moves to deck location...	Action
Addition step 1	1	Washes the syringes.
	1	Sets the Peltier Thermal Station to 60 °C and pauses until the temperature reaches 55 °C.
	5	Aspirates air into the syringes while moving to location 5.
	5	Prewets the syringes by cycling up and down 3 times. Goes to the top of the wells and dispenses air and then performs a tip touch. Aspirates 20 µL of Denaturation Mixture.
	4	Picks up the lid.
	3	Drops off the lid.
	4	Dispenses 20 µL of Denaturation Mixture, and then cycles up and down 15 times to mix the solution. Goes to the top of the wells and dispenses air and then does a tip touch.
	3	Picks up the lid.
	4	Drops off the lid.
	1	Dispenses any air remaining in the syringes, and then washes the syringes. *Note:* The protocol pauses until you click Continue in the Scheduler Paused window.

Protocol step	Head moves to deck location...	Action
Addition step 2	1	Sets the Peltier Thermal Station to 25 °C and pauses until the temperature reaches 30 °C.
	6	Picks up the lid.
	3	Drops off the lid.
	6	Aspirates air into the syringes while moving to location 6.
	6	Prewets the syringes by cycling up and down 3 times. Goes to the top of the wells and dispenses air and then performs a tip touch. Aspirates 5 µL of Alkylant.
	3	Picks up the lid.
	6	Drops off the lid.
	4	Picks up the lid.
	3	Drops off the lid.
	4	Dispenses 5 µL of Denaturation Mixture, and then cycles up and down 15 times to mix the solution. Goes to the top of the wells and dispenses air and then does a tip touch.
	3	Picks up the lid.
	4	Drops off the lid.
	1	Dispenses any air remaining in the syringes, and then washes the syringes.
	1	Waits for 30-minute incubation.

Protocol step	Head moves to deck location...	Action
Addition step 3	1	Sets the Peltier Thermal Station to 25 °C and pauses until the temperature reaches 20–25 °C.
	7	Aspirates air into the syringes while moving to location 7.
	7	Prewets the syringes by cycling up and down 3 times. Goes to the top of the wells and dispenses air and then performs a tip touch. Aspirates 140 µL of Diluent Mixture.
	4	Picks up the lid.
	3	Drops off the lid.
	4	Dispenses 140 µL of Diluent Mixture, and then cycles up and down 15 times to mix the solution. Goes to the top of the wells and dispenses air and then does a tip touch.
	3	Picks up the lid.
	4	Drops off the lid.
	1	Dispenses any air remaining in the syringes, and then washes the syringes.
	1	Waits for 0-minute incubation.

Protocol step	Head moves to deck location...	Action
Addition step 4	1	Sets the Peltier Thermal Station to 37 °C.
	1	Moves the syringes up and down for 4 cycles with a wicking across the chimneys after every cycle.
	2	Mounts the pipette tips on the head.
	8	Aspirates air into the syringes while moving to location 8.
	8	Prewets the pipette tips by cycling up and down 3 times. Goes to the top of the wells and dispenses air and then performs a tip touch. Aspirates 5 µL of Protease.
	4	Picks up the lid.
	3	Drops off the lid.
	4	Dispenses 5 µL of Protease, and then cycles up and down 15 times to mix the solution. Goes to the top of the wells and dispenses air and then does a tip touch.
	3	Picks up the lid.
	4	Drops off the lid.
	1	Dispenses any air remaining in the syringes, and then washes the pipette tips.
	2	Parks the pipette tips in the seating station.
	1	Waits for 180-minute incubation.

Protocol step	Head moves to deck location...	Action
Addition step 5	1	Sets the Peltier Thermal Station to 25 °C.
	9	Aspirates air into the syringes while moving to location 9.
	9	Prewets the syringes by cycling up and down 3 times. Goes to the top of the wells and dispenses air and then performs a tip touch. Aspirates 20 µL of Acidification Reagent.
	4	Picks up the lid.
	3	Drops off the lid.
	4	Dispenses 20 µL of Acidification Reagent, and then cycles up and down 15 times to mix the solution. Goes to the top of the wells and dispenses air and then does a tip touch.
	3	Picks up the lid.
	4	Drops off the lid.
	1	Dispenses any air remaining in the syringes, and then washes the syringes.
	1	Waits for 0-minute incubation.

Reference library

1 Bovee, M., Russel, J., Murphy, S., Automation of Sample Preparation for Accurate and Scalable Quantification and Characterization of Biotherapeutic Proteins Using the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-4872EN, 2016

See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Bravo Platform.

10 On-Cartridge Reaction v2.0 User Guide

This chapter contains the following topics:

App description

On-Cartridge Reaction v2.0. This application enables the automated aspiration of a temperature-controlled reagent, for example, an enzyme solution, through prepared AssayMAP cartridges that contain immobilized target molecules. This allows the separation of reaction products, simplifying downstream sample preparation and analytical processes. The protocol enables processing of from 1 to 96 reactions in a single run.

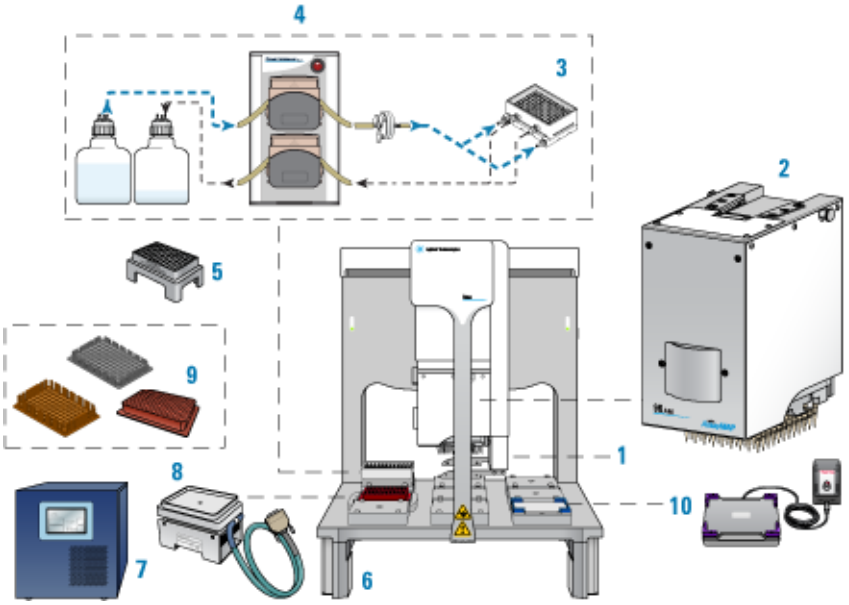
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the On-Cartridge Reaction v2.0 protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the most common AssayMAP cartridges for performing the On-Cartridge Reaction on the AssayMAP Bravo Platform. But any AssayMAP cartridge can be used with this application. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Protein A (PA-W) cartridge rack	G5496-60000	G5496-60018
AssayMAP Protein G (PG-W) cartridge rack	G5496-60008	–
AssayMAP Streptavidin (SA-W) cartridge rack	G5496-60010	G5496-60021
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of Protein A, Protein G, or Streptavidin 5- or 25-µL cartridges are required. For details, see Planning the cartridge layout.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

  Note: The On-Cartridge Reaction app uses cartridges that have been primed in another application. For example, you might run Affinity Purification to immobilize samples on the cartridges that you want to use for On-Cartridge Reaction.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out rapidly if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution. See "How to store prepared cartridges" on page 320 for more details.

- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.

  If cartridge reuse is desired (for example, use of cartridges with immobilized customer-supplied affinity ligands), the customer is responsible for determining acceptable performance. In this case, ensure that you use particulate-free samples, equilibrate the cartridges using a solution compatible with the surface chemistry and resin matrix, and never allow the cartridges to dry out.

- PA-W, SA-W, and PG-W cartridges tolerate brief exposure to pH as low as 2.0. The stability of the PA-W, SA-W, and PG-W cartridges after capturing additional affinity ligands should be determined empirically.

Labware

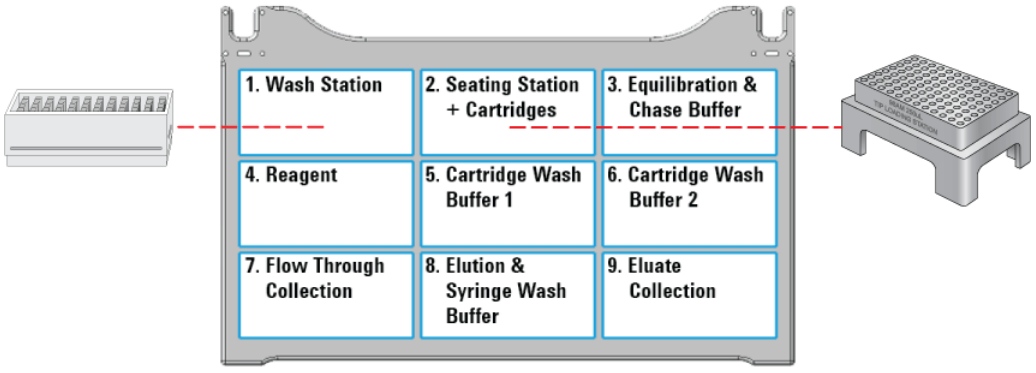
Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3—9
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3—9
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottoms	Agilent 201254-100	3—9
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	3—9
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—9
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	3—9**
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	3—9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	3—9**
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3—9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	3—9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	3—9
96 Red PCR Insert + Eppendorf 30129300, PCR, Full Skirt	Agilent insert (provided) and Eppendorf 30129300	4
96 Red PCR Insert + Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Agilent insert (provided) and Bio-Rad HSP-9611	4**
96 Red PCR Insert + Greiner 652270, PCR, Full Skirt, PolyPro	Agilent insert (provided) and Greiner 652270	4**
96 Greiner U-Bottom Thermal Insert + Greiner 650201_U-Bottom, Clear PolyPro	Agilent G5498B#126 and Greiner 650201	4
96 Greiner V-Bottom Thermal Insert + Greiner 651201 V-Bottom, Clear PolyPro	Agilent G5498B#126 and Greiner 651201	4
96 AbGene U-Bottom Thermal Insert + AbGene 1127, Square Well, Round Bottom	Agilent G5498B#127 and ABgene AB-1127	4

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner and BioRad PCR plates are not compatible with the 25 µL cartridges at deck locations 3, 4, 7, and 9.

Reagents

The volume, type, and concentration of reagents required for On-Cartridge Reaction vary depending on sample characteristics and the desired analytical result. For Agilent application notes and peer-reviewed papers using the On-Cartridge Reaction application, see the "Reference library" on page 353 and the Agilent AssayMAP Bravo Citation Index, respectively. These resources provide detailed examples of reagents used successfully with this application in a variety of workflows.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details about this utility and the reagent recommendations for washing the syringes, see the Syringe Wash v3.0 User Guide.

All labware requires volume overage for the protocol to execute properly. Use the On-Cartridge Reaction Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 315.

Preparing the solutions

The following solutions are required for the On-Cartridge Reaction protocol:

- Equilibration & Chase Buffer
- Reagent (for example, an enzymatic or chemical agent)
- Cartridge Wash Buffers
- Elution & Syringe Wash Buffer

CAUTION

A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for On-Cartridge Reaction

The Reagent Volume Calculator is a Microsoft Excel file that contains a Calculator worksheet. You enter the number of columns to process, whether to perform the Collect Flow Through options, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

To use the Reagent Volume Calculator:

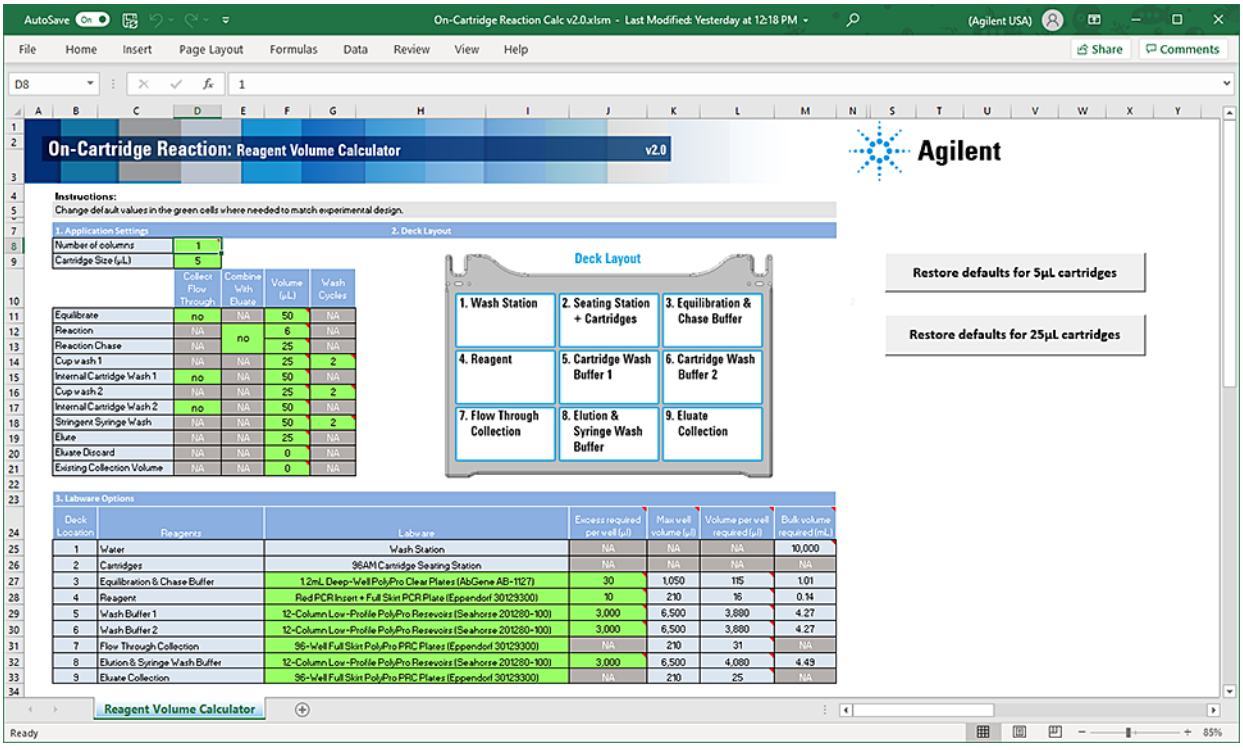
1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figure shows the On-Cartridge Reaction Reagent Volume Calculator.

Figure On-Cartridge Reaction Reagent Volume Calculator



Determining the Reagent volume for deck location 4

The Reagent Volume Calculator determines a recommended volume for the Reagent (deck location 4) based on the reaction volume plus a standard overage for the labware type. The values for both the reaction volume and the overage are influenced by multiple factors.

Note: The labware-based overage in the Reagent Volume Calculator is a general recommendation that can changed. The overage value may be higher or lower than the default based on the length of the protocol run, when the labware is used during the run, and the volatility of the solution used in the labware. The Reagent (deck location 4) has additional factors to consider when determining the overage volume to use.

The Reaction volume is the volume that will be moved over the cartridge during the Reaction step, which is defined by the reaction time input. This volume is drawn up through the cartridges from the wells in two stages of the reaction:

1. First stage. The Initial Draw volume is drawn up through the cartridges at a relatively rapid rate to quickly replace the volume in the resin bed with the reaction solution (for example, enzyme solution).

   Agilent recommends using the default value for the Initial Draw: 4 µL (5 µL cartridges) or 20 µL (25 µL cartridges). The default value is slightly less than one column volume, which should be sufficient for replacing the dead volume in the resin bed.

2. Second stage. The difference between the Reaction volume and the Initial Draw is aspirated over the resin bed at a flow rate that is required for this volume to be aspirated through the resin bed for the Duration input on the form.

The Reaction volume cannot be less than the Initial Draw volume, but it can be equal to it. How much larger the Reaction volume is compared to the Initial Draw is dependent on how much volume one wants to draw over the resin bed during the reaction. The properties of the reaction (for example, stability of the reacting agent, cost, molar ratio of reactant to substrate required, and so forth) will drive this decision.

When choosing an overage volume, consider the following:

- Dead volume required to ensure that air is not aspirated into the cartridge
- Evaporation, which is dependent on the temperature of the reaction
- Length of the reaction and the volatility of the sample during the sample loading step

Enough liquid must remain in the wells to act as a heat conductor to the resin bed. At least 3–5 µL of liquid is required in each well of a 96-well PCR plate at the end of the incubation time, as this is the minimum volume required to maintain contact with the bottom of the cartridges. This contact is required to conduct heat into the resin bed. A PCR plate is the most practical and common labware type at deck location 4 because of the low volumes of solution typically used at that location and the low dead volume requirements for a PCR plate.

Example using 5 µL cartridges

Note: See the app notes in the "Reference library" on page 353.

In this example, a PCR plate is seated in the Red PCR Plate Insert at deck location 4. To conduct a 30-minute reaction at a resin bed temperature of approximately 37 °C with a 6 µL total aspiration volume, the temperature should be set to 45 °C and 12 µL of enzyme solution should be added per well before the run is started. A volume of 12 µL is required in this example because:

- 6 µL will be aspirated (4 µL initial draw and 2 µL drawn through the resin bed over the 30 minute reaction).

- 3 µL is expected to evaporate.
- 3 µL is required at the end of the run to ensure heat conductance occurs during the entire run.

For more details, see the Reaction Volume and Temperature steps in "Assay development guidelines and protocol notes" on page 333.

Preparing the buffers and reagents

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent Technologies recommends the buffers listed in the following table as a **starting point** for optimizing the AssayMAP On-Cartridge Reaction chemistry.

Table Reagent preparation

Reagent and deck location	Composition and comments
Equilibration & Chase Buffer (deck location 3)	This reagent should provide optimal conditions for the reaction step, so it is typically the solvent used for the reaction reagent. For an enzymatic On-Cartridge Reaction run, this would typically be the enzyme reaction buffer. This buffer should not disrupt the affinity interaction between the cartridge resin and immobilized substrate.
Reagent (deck location 4)	Contains molecules that modify the immobilized substrate during the on-cartridge reaction. • Enzymatic reaction, this solution would contain the enzyme. • Chemical reaction, this solution would contain chemically active molecules.
Cartridge Wash Buffer 1 (deck location 5)	High-stringency buffer (for example, a neutral buffer with high NaCl) or a low-stringency buffer (for example, a neutral, mass-spec-compatible buffer). The On-Cartridge Reaction protocol is designed to allow up to two sequential washes. The buffer selection depends on a number of factors, such as: • Whether Wash Buffer 2 will be used, which is often dependent on whether a stringent wash is desired in the protocol. If so, Wash Buffer 1 would be a stringent buffer, and Wash Buffer 2 would be a less stringent wash buffer, which would remove the stringent component of the buffer before elution. • If a stringent buffer is not used, typically, you would use Wash Buffer 1 only. The buffer would be a low-stringency wash buffer, for example, a neutral mass-spec-compatible buffer.

Reagent and deck location	Composition and comments
Cartridge Wash Buffer 2 (deck location 6)	Low-stringency buffer (for example, a neutral, mass-spec-compatible buffer). The composition of this buffer is often dictated by sensitivity of downstream steps to components of Cartridge Wash Buffer 2, as the void volume in the cartridges (~2 µL) will contain the last wash solution used before the elution step and end up in the eluate unless the Eluate Discard option is selected in the elution step.
Elution & Syringe Wash Buffer (deck location 8)	Typically a low pH, mass-spec friendly solution, but this is highly dependent on the nature of the affinity interaction to be broken. A key consideration is if the sample will be neutralized following elution. If so, a weak acid that is easy to neutralize would be a good choice (for example, 12 mM HCl with 100 mM NaCl for the interaction between protein A and an antibody). However, this type of elution solution may be relatively inefficient in breaking the affinity interaction and, therefore, may require more volume for complete elution (for example, 4–6 column volumes for complete elution of antibodies off a protein A cartridge). If neutralization is not a key consideration or minimizing elution volume is a critical driver, a strong acid is a better choice (for example, 5% acetic acid) because complete elution can be achieved in as little as 2–3 column volumes.

When preparing these reagents, you must:

- Remove macromolecular particulates.
- Adjust the buffer composition to optimize the pH conditions.
- Determine the volume of solutions required using the Reagent Volume Calculator.

The prepared reagents should be kept in a closed container before use to avoid evaporation.

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware columns or wells that correspond with the cartridge positions on the Cartridge & Tip Seating Station. For more information, see "Planning the cartridge layout" on page 321.

To dispense the reagents into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Equilibration & Chase Buffer into the labware to be placed at deck location 3.
3. Add the specified volume of Reagent into the labware to be placed at deck location 4.

4 Add the specified volume of Cartridge Wash Buffer 1 into the labware to be placed at deck location 5.

5 If using two wash buffers, add the specified volume of Cartridge Wash Buffer 2 into the labware to be placed at deck location 6.

6 Add the specified volume of Elution & Syringe Wash Buffer into the labware to be placed at deck location 8.

7 If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can run the Reagent Aliquot utility or Reagent Transfer utility to transfer the buffers and reagents to different labware. For instructions, see the "Reagent Aliquot v2.0 User Guide" on page 518 or "Reagent Transfer v3.0 User Guide" on page 525.

Preparing cartridges with immobilized substrate

How to prepare the cartridges

The On-Cartridge Reaction app facilitates reactions between a substrate immobilized on a cartridge and a soluble reactant. The cartridges containing the immobilized substrate are prepared before the On-Cartridge Reaction run using any of the other AssayMAP applications, typically, Affinity Purification.

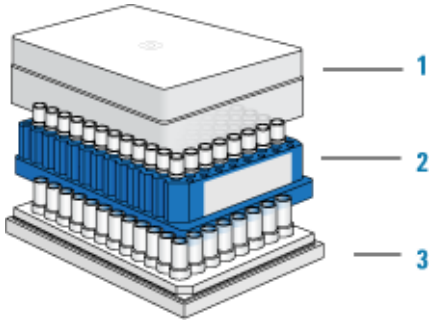
How to store prepared cartridges

IMPORTANT

Do not allow cartridges with immobilized substrate to dry out. If the cartridges do dry out, do not use them.

To avoid cartridges drying out, store them immediately after the Affinity Purification run in an AssayMAP cartridge rack and receiver plate assembly, where each chimney of the receiver plate housing a cartridge with an immobilized substrate contains 200 µL of buffer. Use a buffer that is known to be compatible with the cartridge's immobilized substrate and resin.

Figure AssayMAP cartridge rack lid (1), rack with cartridges (2), and receiver plate (3)



Using this storage method, the tip of the cartridge is submerged in a buffer and prevents the cartridge bed from drying out. Place the lid on the cartridge rack and receiver plate stack to prevent anything falling into the cartridges. For storage longer than a few hours, the assembly should be stored in a refrigerator (4–10 °C).

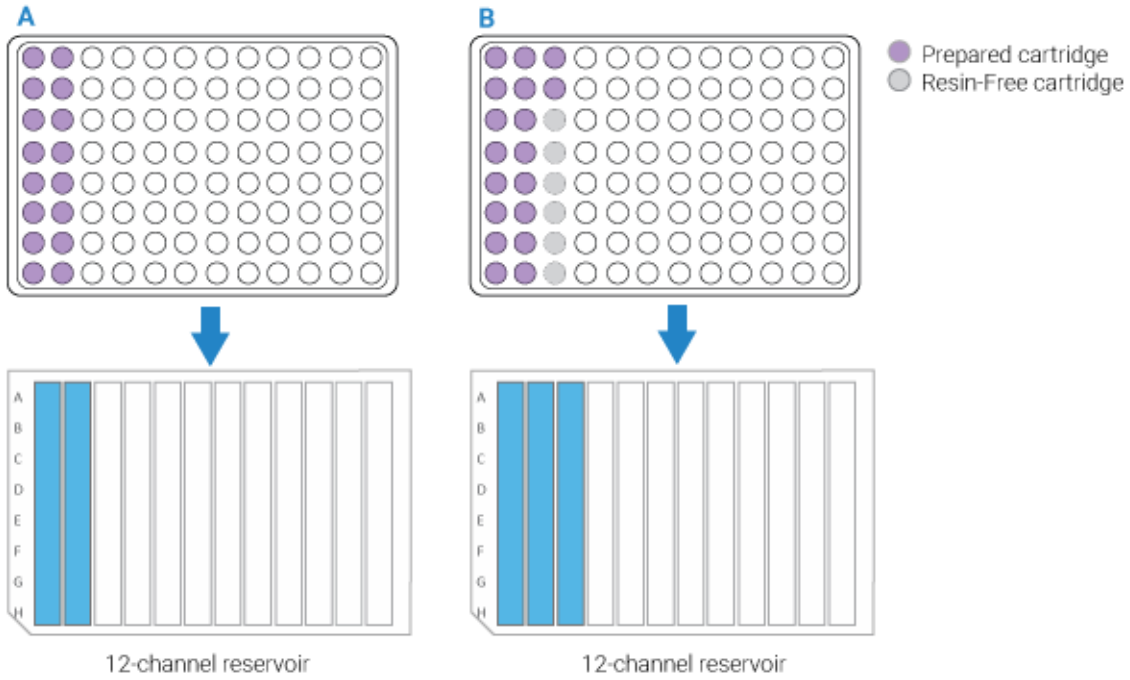
Planning the cartridge layout

You can process 1 to 96 cartridges with immobilized substrate in parallel. The position of the cartridges in the seating station dictates the position of the reagent and buffer solutions in the microplates at each deck location.

- If you have fewer than 96 cartridges, make sure the cartridges occupy full columns in the microplate, as the figure below shows.

  The default protocol settings assume that cartridges will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based on the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of cartridges you have is not a multiple of 8, use AssayMAP Resin-Free cartridges to fill the empty well positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash steps.

Figure Examples of microplate and reservoir layout: A) Multiple of 8 samples, B) Not a multiple of 8 samples



The positions of the cartridges at deck location 2 dictate the positions of the solutions in deck locations 3, 4, 5, 6, and 8. See "Labware" on page 313 for the acceptable labware at each deck location.

Running the protocol

The On-Cartridge Reaction protocol does the following:

- Washes the syringes.
- Equilibrates the cartridges.
- Loads the Reaction reagent onto the cartridges and incubates the reaction at the temperature set point.
- Collects soluble reaction products.
- Removes non-target elements from the immobilized reaction product.
- Elutes the immobilized reaction product from the cartridges.

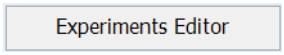
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the Cartridge & Tip Seating Station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 315.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup utility to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

  This application uses cartridges that have been prepared during a preceding AssayMAP application, typically, Affinity Purification.

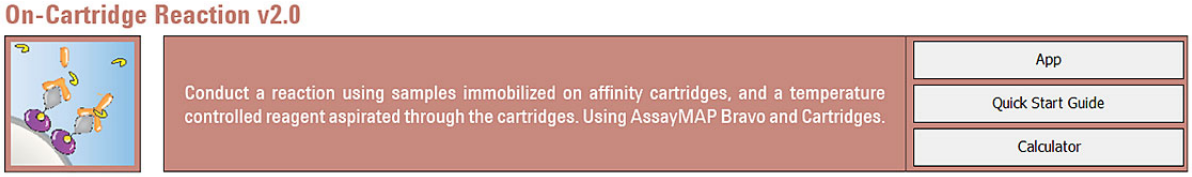
IMPORTANT Do not allow cartridges with immobilized substrate to dry out. If the cartridges do dry out, do not use them.

Setting up the protocol

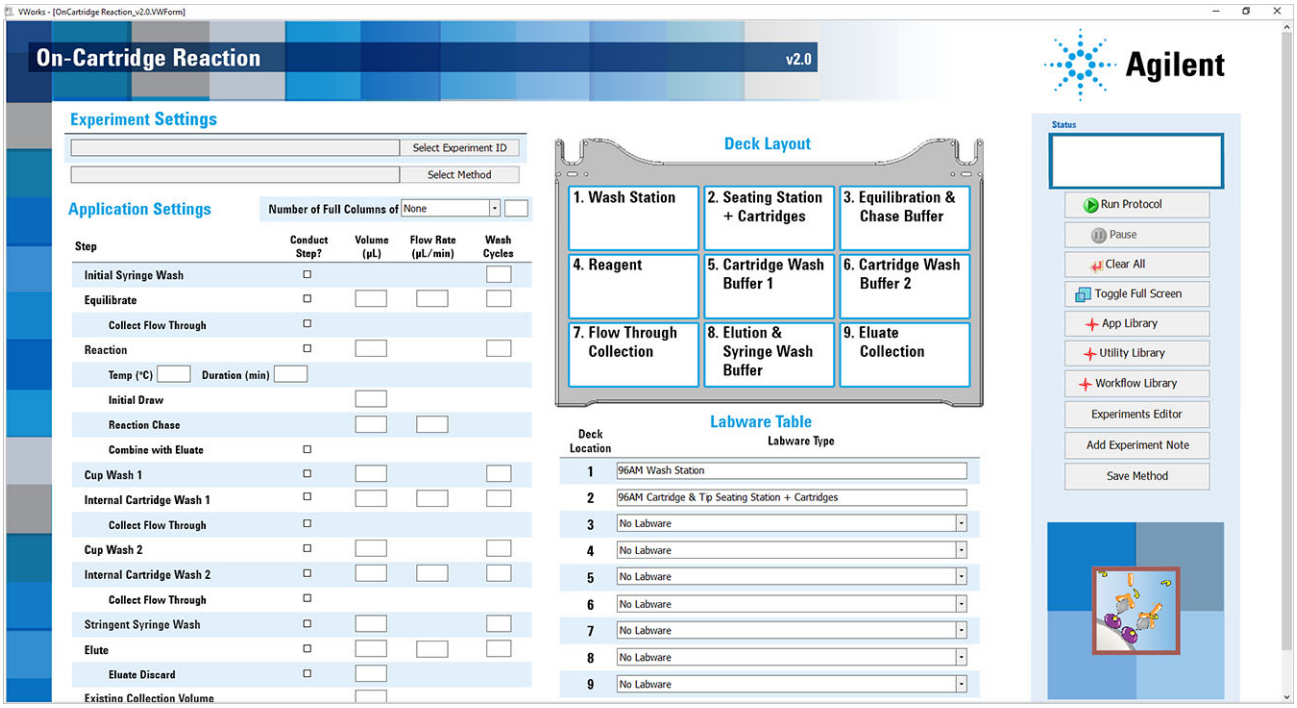
Before starting the protocol, make sure the appropriate selections and values are specified in the On-Cartridge Reaction application.

To set up the protocol:

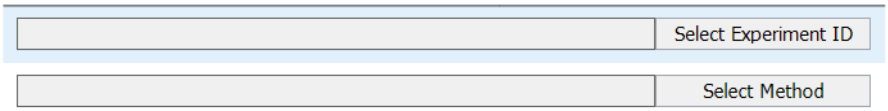
1. Open the **App Library**.
2. Locate **On-Cartridge Reaction v2.0**, and then click **App**.



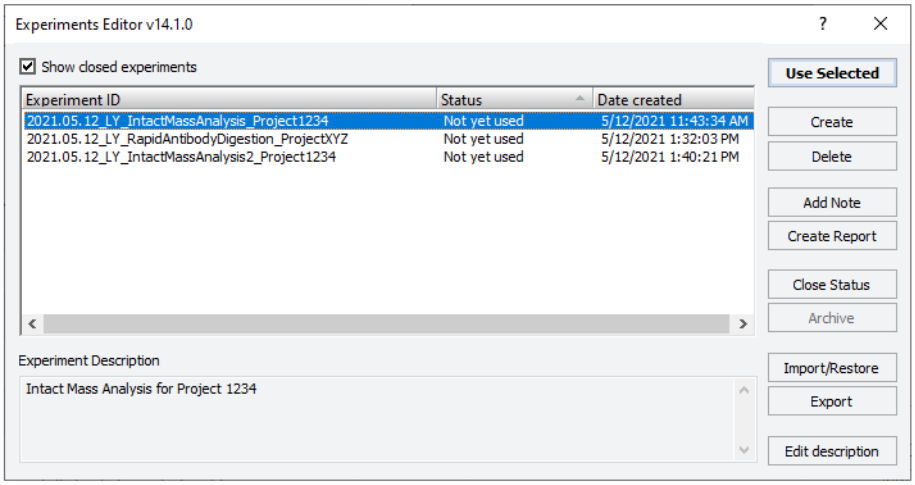
The On-Cartridge Reaction application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

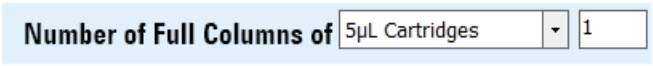
5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 330.
- To create or modify the method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:



a Select the cartridge size from the list:
- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION

If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.

CAUTION

If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.

IMPORTANT

Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the full and practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 333.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Equilibrate	Aspirates the Equilibration Buffer (deck location 3) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1) or into Flow Through Collection (deck location 7).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Equilibrate flow-through at Flow Through Collection (deck location 7). If not selected, discards the equilibration flow-through at the wash station (deck location 1).	–	–	–	–
Reaction	Aspirates the Reagent (deck location 4) through the cartridges in two steps (see note), followed by aspirating a chase volume. The Reaction flow-through and chase volume are collected at Flow Through Collection (deck location 7), unless Combine with Eluate is selected. The sum of the Reaction volume and the Reaction Chase volume must be less than 250 µL.	5 µL:	6	See note.	3
		25 µL:	30		3
		Range:	0–250		0–10
	Note: The initial draw volume is aspirated at 10 µL/min. Any additional volume (reaction volume minus the initial draw volume) is aspirated at a flow rate appropriate to satisfy the Duration setting.				

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Tempera-ture	Specifies the set point temperature of the Peltier Thermal Station at deck location 4 during the Reaction step. The temperature in the cartridge will be less than this setting.	5 µL:	Temperature: 25 °C		
		25 µL:	25 °C		
		Range:	4–110 °C		
Duration	Specifies the total length of time to aspirate the Reagent (deck location 4) through the cartridges.	Range:	Time (m): 30 (0–180)		
Initial Draw	Specifies the initial draw volume, which is aspirated at 10 µL/min. *Note:* Any additional volume (reaction volume minus the initial draw volume) is aspirated at a flow rate appropriate to satisfy the Duration setting.	5 µL:	4	10	–
		25 µL:	20	10	–
		Range:	0–250	–	–
Reaction Chase	Aspirates the Chase Buffer (deck location 3) through the cartridges, to flush soluble reaction products into the syringes. This step occurs immediately after the aspiration of Reagent, combining the soluble reagent products and chase buffer within the syringes.	5 µL:	15	5	–
		25 µL:	75	5	–
		Range:	0–250	0.1–500	–
Combine with Eluate	If selected, collects the soluble reaction products at Eluate Collection (deck location 9). If not selected, collects the soluble reaction products at Flow Through Collection (deck location 7).	–	–	–	–
Cup Wash 1	Rinses the cartridge cups with the Cartridge Wash Buffer 1 (deck location 5), and then discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 1	Aspirates the Cartridge Wash Buffer 1 (deck location 5) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1) or Flow Through Collection (deck location 7).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash 1 flow-through at Flow Through Collection (deck location 7). If not selected, discards the Internal Cartridge Wash flow-through at the wash station (deck location 1).	–	–	–	–

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Cup Wash 2	Rinses the cartridge cups with the Wash Buffer 2 (deck location 6) and discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash 2	Aspirates the Cartridge Wash Buffer 2 (deck location 6) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1) or Flow Through Collection (deck location 7).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash 2 flow-through at Flow Through Collection (deck location 7). If not selected, discards the Internal Cartridge Wash 2 flow-through at the wash station (deck location 1).	–	–	–	–
Stringent Syringe Wash	Aspirates the Syringe Wash Buffer (deck location 8), and then discards the liquid into the wash station (deck location 1).	5 µL:	50	–	2
		25 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates the Elution Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into Eluate Collection (deck location 9).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0–250	0.1–500	0–10
Eluate Discard	If selected, a specified initial volume of Eluate will be dispensed through the cartridges, and then discarded at the wash station (deck location 1).	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–250	–	–
Existing Collection Volume	Specifies the volume of liquid present in the Eluate Collection plate (deck location 9) at the beginning of the run.	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–1000	–	–
Final Syringe Wash	Conducts the specified number of internal syringe washes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 333.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 349.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT The protocol will display an error message if cartridges are missing.

Starting the protocol run

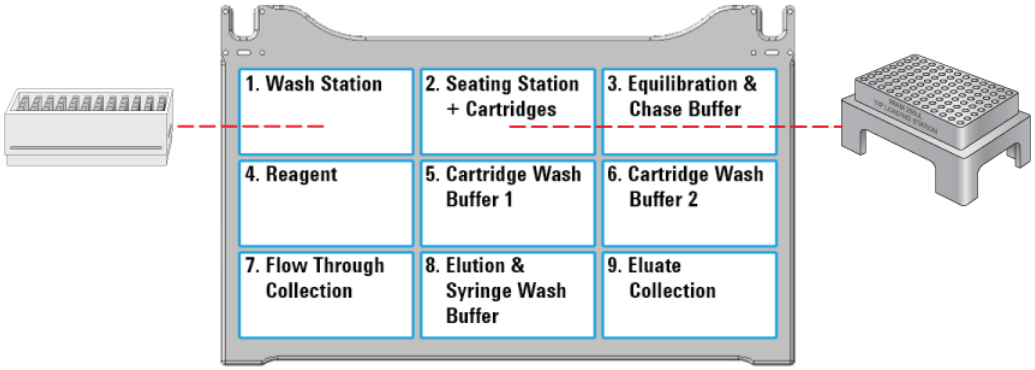
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

Note: The Greiner and BioRad PCR plates are not compatible with the 25 µL cartridges at deck locations 3, 4, 7, and 9.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

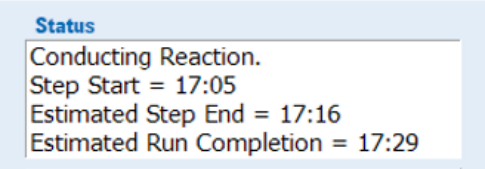
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING

To stop a run in an emergency, use the emergency stop pendant.

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

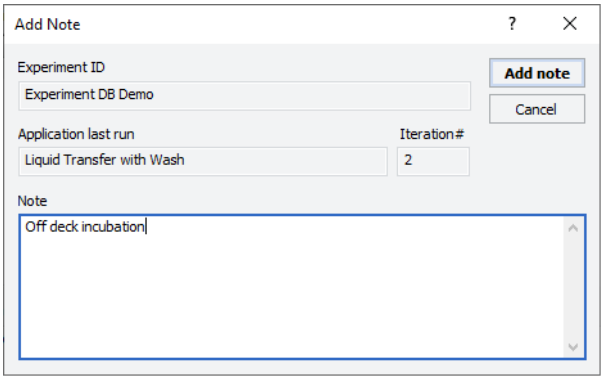
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



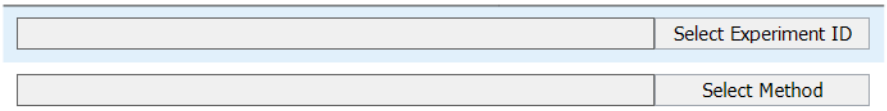
2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

1. Remove used labware from the deck.
2. Discard leftover reagents appropriately.
3. *Optional*. Conduct stringent washing of the syringes:
   a. Open the **Syringe Wash** utility  .
   b. If applicable, click **Select Experiment ID** to open the Experiments Editor.



   c. In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.
   d. Click **Select Method** to select and load the method for this utility.
   e. Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.
   f. Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the On-Cartridge Reaction protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 10 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12

Protocol step	Guidelines and notes
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for the reaction during the Reaction step. In the case of an enzymatic reaction, the equilibration buffer would be the optimized enzyme reaction buffer. Ensure that the optimal reaction buffer does not break the affinity interaction holding the immobilized substrate on the resin bed. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow rate**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min using the default volume may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	This step is used for optimizing or troubleshooting a protocol to ensure that the equilibration solution is not eluting the immobilized substrate off the cartridge. If this step is selected, the dispenses the flow-through during the Equilibrate step into the Flow Through Collection plate for downstream processing or for analysis. If this step is not selected, the flow-through from the Equilibrate step is dispensed directly into the wash station. This step is not selected by default.
Reaction	This step enables the reaction in the cartridge resin bed. No liquid is removed or added to the cartridge cups before the reaction begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Reaction step aspirates the Reagent through the cartridges in two steps, and then aspirates a chase volume: **1** Initial Aspiration. Aspirates the Initial Draw volume at a flow rate of 10 µL/min. **2** Secondary Aspiration. Aspirates any additional volume at a flow rate appropriate to satisfy the Duration setting. For example, if the volume is set at 6 µL and the time was set for 30 minutes, the remaining 2 µL of the 6 µL volume would be aspirated at a flow rate of ~0.068 µL/min (2 µL / (30 min – 0.4 min)). **3** Reaction Chase Aspiration. Aspirates the chase at the specified volume and flow rate. The Reaction flow-through and Reaction Chase are collected in the syringes, and then dispensed into the Flow Through Collection plate, unless Combine with Eluate is selected. In which case, the Reaction flow-through and the Reaction Chase are dispensed into the Elution plate. This step is selected by default.

Protocol step	Guidelines and notes
Reaction (continued)	**Volume (µL).** The volume must be determined empirically. For an enzymatic reaction, the volume would depend on the enzyme. If the enzyme is robust enough to undergo the number of enzymatic cycles required to push the reaction to completion, the Initial Draw volume may be sufficient. If the enzyme undergoes a limited number of cycles, the enzyme in the resin bed may need to be replenished during the course of the reaction, and significantly more than the Initial Draw volume may be required. • Volume for 5 µL cartridge: – Default: 6 – Practical: 4–15 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 30 – Practical: 20–75 – Range: 0–250 **Flow Rate.** The initial draw volume is aspirated at 10 µL/min. Any additional volume (reaction volume minus the initial draw volume) is aspirated at a flow rate appropriate to satisfy the Duration setting. **Wash Cycles**: The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
	Scenario using default settings for 5 µL cartridges: **1** The Peltier Thermal Station heats until the set point temperature ±2°C is reached. **2** The Bravo 96AM Head picks up the cartridges from the Cartridge & Tip Seating Station. **3** The Reagent is aspirated through the cartridges: 4 µL at a flow rate of 10 µL/min and 2 µL at a flow rate of 0.068 µL/min. (The Duration of both aspiration steps is 30 minutes.) **4** An external cartridge wash is conducted at the wash station. **5** 15 µL Chase Buffer is aspirated at 5 µL/min. **6** The cartridges are ejected into the Cartridge & Tip Seating Station. **7** The syringe contents are dispensed into the Flow Through Collection plate. **8** Three internal syringe wash cycle are conducted.

Protocol step	Guidelines and notes
Temperature	This setting specifies the set point temperature of the Peltier Thermal Station during the Reaction step. The optimal temperature is a function of the reaction being conducted. The actual temperature in the cartridge will be less than this setting. Heat loss occurs as the heat transfers across the thermal plate insert, a small air gap between the plate insert and the wells of the plate, the plastic of the plate, the solution in the well, and the cartridge resin bed. For example, a temperature setting of 45 °C results in a cartridge bed resin temperature of approximately 37 °C using the Red PCR Plate Insert and a PCR plate. A thermal plate insert is critical when running a reaction at a temperature other than room temperature. Without the insert, the air gap between the Thermal Station and the wells of the plate results in a greater temperature differential between the setting in the application and the actual temperature in the cartridge bed. The temperature differential is not a constant percentage of the set point, so the preceding example should be considered only as a starting point for cartridge target temperatures other than 37 °C in the PCR plates. In addition, plate types vary in heat transfer efficiency because of their differences in design, such as well shape. **IMPORTANT** At least 3–5 µL of liquid is required in each well of a 96-well PCR plate at the end of the incubation time as this is the minimum volume required to maintain contact with the bottom of the cartridges. This contact is required to conduct heat into the resin bed. Longer incubation times or higher temperature settings require more volume at the beginning of the run to account for added evaporation. This volume should be determined empirically. A PCR plate is the most practical and common plate type at deck location 4 because of the low volumes of solution typically used at that location. The minimum volume required in the wells at the end of the incubation time for other plate types must be determined empirically. **Example using Red PCR Plate Insert plus PRC plate**. To conduct a 30-minute reaction at a resin bed temperature of approximately 37 °C with a 6 µL total aspiration volume, the temperature should be set to 45 °C and 12 µL of enzyme solution should be added per well before the run is started. 12 µL is required because 6 µL will be aspirated, 3 µL are expected to evaporate, and 3 µL are required at the end of the run to ensure heat conductance occurs during the entire run. **Temperature °C:** • Default: 25 • Practical: 20–60 • Range: 4–110

Protocol step	Guidelines and notes
Duration	This setting specifies the total length of time to aspirate the Reagent through the cartridges, including the initial and secondary aspiration steps. *Note:* This setting governs only the aspiration of Reagent through the cartridges. It does not include the time required to ramp up to temperature or the time required for the Reaction Chase step. *Note:* The duration of the reaction is related to the concentration of the reactant. In general, as the concentration of the reactant (for example, enzyme) decreases, the duration increases, as does the amount of reaction solution lost during the reaction due to evaporation, and the volume of excess reaction solution required. See the notes in the preceding Temperature step for more details. **Time (minutes):** • Default: 30 • Practical: 5–60 • Range: 0–180
Initial Draw	Specifies the initial draw volume, which is aspirated at 10 µL/min. Any additional volume (reaction volume minus the initial draw volume) is aspirated at a flow rate appropriate to satisfy the Duration setting. **Volume (µL):** • Volume for 5 µL cartridge: – Default: 4 – Practical: 3–9 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 20 – Practical: 15–45 – Range: 0–250 *Note:* Setting the volume to zero skips all Reaction tasks except syringe washing.

Protocol step	Guidelines and notes
Reaction Chase	This step washes the soluble reaction products out of the resin bed and into the syringes using the Chase Buffer. The Reaction Chase occurs immediately after the aspiration of Reagent, combining the flow-through of the initial and secondary aspiration and the Chase Buffer within the syringes. **Volume (µL).** Elution of products off the AssayMAP cartridges is typically 2–3 column volumes when the affinity between the eluate and resin has been completely disrupted. Therefore, 10–15 µL (5 µL cartridge) or 50–75 µL (25 µL cartridge) should be a sufficient chase volume as long as there is no significant affinity between the soluble reaction product and the resin bed. The default volume is set to 3 column volumes to be conservative, but it is likely that you can decrease this volume. • Volume for 5 µL cartridge: – Default: 15 – Practical range: 15–30 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 75 – Practical range: 50–100 – Range: 0–250 *Note:* Setting the volume to zero skips all Reaction tasks except syringe washing. **Flow Rate:** • Default: 5 • Practical: 5–20 • Range: 0.1–500
Combine with Eluate	This setting specifies where the combination of the Reaction initial and secondary aspiration and the Chase Buffer is collected: • If selected, collects the Reaction initial and secondary aspiration and chase in the Eluate Collection plate. This is a good choice if the downstream analysis can easily separate the reactant, soluble, and eluted reaction products, allowing all the reaction products to be analyzed in a single run. • If not selected, collects the Reaction initial and secondary aspiration and chase in the Flow Through Collection plate. This is a good choice if the goal is to analyze the soluble and immobilized reaction products separately because – Data on only one of these components is required, and there is not a good or rapid way of chromatographically separating the reactant, buffer, soluble, and immobilized reaction products in a single analytical run. – One wants to remove the buffer, reactant, and soluble reaction product from the immobilized reaction product, and elute this immobilized reaction product in a mass-spec-compatible solution. The setting is not selected by default.

Protocol step	Guidelines and notes
Cup Wash 1	This step removes the residual liquid that may remain above the resin bed after the Reaction step. The Cup Wash 1 step aspirates Cartridge Wash Buffer 1 into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the wash station, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 1	This step uses Cartridge Wash Buffer 1 to wash non-specifically bound molecules from the resin bed. In preparation for Internal Cartridge Wash 1, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Cartridge Wash Buffer 1 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 1 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	This step is typically used for optimization or troubleshooting of a protocol to ensure that the wash solution is not eluting the immobilized substrate off the cartridge. If this step is selected, the flow-through from Internal Cartridge Wash 1 step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from Internal Cartridge Wash 1 is dispensed into the wash station. This step is not selected by default.
Cup Wash 2	This step removes the residual buffer that may remain above the resin bed after the Internal Cartridge Wash 1 step. This step aspirates Cartridge Wash Buffer 2 and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the previous cartridge wash is aspirated from the cartridge cups. Any cartridges that stuck to the probes during the cup wash are parked at the seating station, and then the liquid in the syringes is dispensed into the wash station. The syringes are washed at the wash station. This step is not selected by default. **Volume (µL)**. A volume less than the default may be insufficient for cup washing, while a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash 2	This step uses Cartridge Wash Buffer 2 to wash non-specifically bound molecules and Cartridge Wash Buffer 1 from the resin bed. In preparation for Internal Cartridge Wash 2, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid that is in the cups is removed by a 60 µL aspiration, the aspirated solution is discarded at the wash station, 10 µL of Cartridge Wash Buffer 2 is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer 2 into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges at the specified flow rate into the Flow Through Collection plate or wash station. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer from the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. This step is not selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) has little benefit but will also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–150 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of deionized water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	This step is typically only used for optimization or troubleshooting of a protocol to ensure that the wash solution is not eluting the immobilized substrate off the cartridge. If this step is selected, the liquid eluted during Internal Cartridge Wash 2 is dispensed directly into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through is dispensed directly into the wash station. This step is not selected by default.
Stringent Syringe Wash	This step cleans the syringes with the Elution Buffer prior to elution. The Stringent Syringe Wash step aspirates the Elution Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the wash station. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume are unlikely to improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash cycle**. A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Elute	This step uses Elution Buffer to elute immobilized reaction products from the cartridges. In preparation for elution, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station, 10 µL of Elution Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges, and then the cartridges are parked at the seating station. After the elution, the eluate is mixed in the Eluate Collection plate and the syringes are washed at the wash station. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples will not be mixed. You can also select the Eluate Discard and Existing Collection Volume substeps, which are described in the following rows of this table. This step is selected by default. **Volume (µL)**. The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges, or 50–75 µL for 25 µL cartridges). The default volumes are conservative and significantly higher than the minimum expected with a strong Elution Buffer. *Note:* The Eluate Collection plate must be able to accommodate the total volume, which is determined by summing the net elution volume (Elute volume - Eluate Discard volume), the Reaction and Reaction chase volumes if Combine With Eluate Volume is selected, and the Existing Collection Volume. For labware-specific maximum well volumes, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. • Volume for 5 µL cartridges: – Default: 25 – Practical: 10–30 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 125 – Practical: 50–150 – Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing.

Protocol step	Guidelines and notes
	Flow rate. A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more elution buffer may be required to get the same elution yield at high elution flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 2–15 • Range: 0.1–500 **Wash cycle**. The number of syringe washes to perform at the wash station after an Elute step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10
Eluate Discard	This substep of the Elute step permits a specified volume of the eluate from the cartridges to be discarded before the eluate starts to be collected during the Elute step. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the Elution Buffer at the Elute flow rate through the cartridges. The Eluate Discard volume is dispensed into the wash station. The remaining Elution Buffer is dispensed through cartridges at the Elute flow rate into the Eluate Collection plate. **Example**: If the Elute and Eluate Discard steps are selected with the following settings, Elute volume = 15 µL (5 µL cartridges) or 50 µL (25 µL cartridges) Eluate Discard volume = 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) the first 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) eluate from the cartridges will be discarded to the wash station, and the remaining 13 µL (5 µL cartridges) or 40 µL (25 µL cartridges) eluate will be collected in the Eluate Collection plate. Select the Eluate Discard step in a situation where minimizing the volume of eluate is critical. For AssayMAP cartridges, the initial elution volume (~2 µL for 5 µL cartridges and ~10 µL for 25 µL cartridges) contains small or no measurable amounts of analyte. This step is not selected by default. **Volume (µL)**. The first volume of eluate that will be discarded during the Elute step. This value can equal, but cannot exceed the Elute volume. • Default: 0 • Practical: – 5 µL cartridges: 0–2 – 25 µL cartridges: 0–10 • Range: 0–250

Protocol step	Guidelines and notes
Existing Collection Volume	This step enables you to specify an amount of liquid that is in the wells of the Eluate Collection plate at the beginning of the run. The Existing Collection Volume, the net volume from the Elute step (Elute volume - Eluate Discard volume), and the soluble reaction products, if Combine With Eluate is selected, feeds into logic that adjusts the well-bottom offset for sample elution, calculates the eluate mixing volume, and dynamically moves the head into and out of the wells during elution and eluate mixing in a volume-dependent manner. For the maximum practical working volumes of labware for eluate collection, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. Select this step when the Eluate Collection plate contains a volume of liquid useful for immediately diluting the eluates, adjusting the pH of the eluates, or to aid in the recovery of small volumes of eluates from AssayMAP cartridges. **Volume (µL):** • Default: 0 • Practical: 0–250 • Range: 0—1000 *Note:* Total elution collection well volumes >500 µL may require additional off-deck mixing to reach homogeneity.
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the wash station. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, the 10 µL of elution buffer will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the On-Cartridge Reaction protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Start protocol	–	Sets Peltier Thermal Station to set point temperature (°C).
	2	Parks any cartridges that may have been mounted on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes the specified number of cycles.
Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then performs an external probe wash.
	3	Aspirates 10 µL of Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Equilibration Buffer into the cartridge cups and exercises the cartridges off task.
	3	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	1	Dispenses the Equilibration Buffer through the cartridges to equilibrate. Washes the cartridge exteriors.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Reaction	1	Waits for Peltier Thermal Station to reach ±2 °C of the Reaction Temperature setting.
	2	Mounts the cartridges on the head.
	4	Aspirates Initial Draw volume at 10 µL/min.
	4	Aspirates the remaining volume (Reaction Volume setting in the form minus Initial Draw volume) for the specified Duration (minutes) at the set Temperature (°C).
	1	Washes the cartridge exteriors at the wash station.
	3	Aspirates the specified volume of Chase Buffer at the specified Flow Rate (µL/min).
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	7	Dispenses the flow-through into the Flow Through Collection plate. Mixes the flow-through.
	1	Washes the syringes.
Cup Wash 1	5	Aspirates cartridge Cartridge Wash Buffer 1 into the syringes.
	2	Washes the cartridge cups and exercises the cartridges off task.
	1	Dispenses the buffer into the wash station between the chimneys.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash 1	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then performs an external probe wash.
	5	Aspirates 10 µL of Cartridge Wash Buffer 1 for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	5	Aspirates Cartridge Wash Buffer 1 into the syringes for the Internal Cartridge Wash 1 step.
	2	Mounts the cartridges on the head.
	1	Dispenses Cartridge Wash Buffer 1 through the cartridges at the Internal Cartridge Wash 1 flow rate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Stringent Syringe Wash	8	Aspirates the Syringe Wash Buffer (Elution Buffer).
	1	Dispenses the buffer at the wash station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Elute	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys, and then performs an external probe wash.
	8	Aspirates 10 µL of Elution Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Elution Buffer.
	2	Mounts the cartridges.
	9	Elutes the samples into the Eluate Collection plate.
	1	Washes the cartridge exteriors.
	2	Parks the cartridges in the seating station.
	9	Mixes eluates.
	1	Washes the syringes.
Final Syringe Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	1	Dispenses into the wash station between the chimneys.
	1	Washes the syringes.

Reference library

1 Han, J., Van Den Heuvel, Z., & Murphy, S. A streamlined drug-to-antibody ratio determination workflow for intact and deglycosylated antibody-drug conjugates, Agilent Technologies, Inc, September 2019, 5991-9010EN.

2 Wu, S., Shen, M., Murphy, S., & Van Den Heuvel, Z. An Integrated Workflow for Intact and Subunits of Monoclonal Antibody Accurate Mass Measurements. Agilent Technologies, Inc, March 2018, 5991-8445EN.

See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Bravo Platform.

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11 Peptide Cleanup v4.0 User Guide

This chapter contains the following topics:

To view the quick start guide for this application, see "Quick start guides" on page 1.

Note: This section presents instructions for using the Peptide Cleanup v4.0 application. If you are using the Aspiration Mode version, see "Peptide Cleanup: Aspiration Mode v3.0 User Guide" on page 393.

App description

Peptide Cleanup v4.0. This application enables automated cleanup of from 1 to 96 peptide samples in a single run.

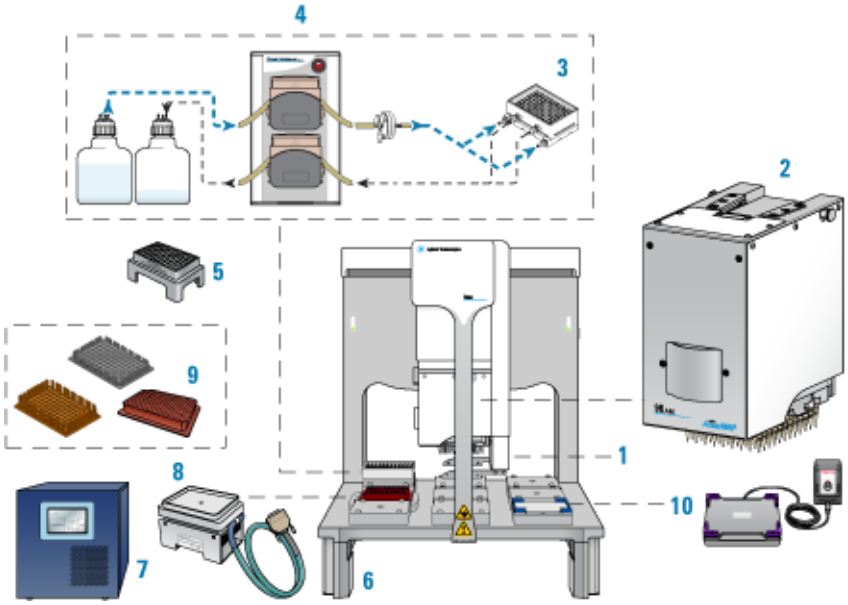
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Peptide Cleanup protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. You might need the following when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate vacuum concentrator for concentration and drying of samples
- Microplate sealer, such as the PlateLoc Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Peptide Cleanup on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Reversed-Phase (C18) cartridge rack	5190-6532	G5496-60017
AssayMAP Reversed-Phase (RP-S) cartridge rack	G5496-60033	G5496-60023
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of C18 and RP-S 5- or 25-µL cartridges are required. For details, see "Preparing the samples" on page 364.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.

Starter kits

The following table lists the two starter kits that are available. Each starter kit contains both cartridges and labware.

Starter Kit	Part number
AssayMAP Digestion and RP-S Cleanup Starter Kit Contains 96 Reversed-Phase (RP-S) cartridges and Labware for In-Solution Digestion: Multi-Plate and Peptide Cleanup	G5496-60034
AssayMAP Digestion and C18 Cleanup Starter Kit Contains 96 Reversed-Phase (C18) cartridges and Labware for In-Solution Digestion: Multi-Plate and Peptide Cleanup	G5496-60013

The following table lists labware that are included in the Peptide Cleanup Starter Kit.

Note: The labware included in the starter kits are for both the Peptide Cleanup and In-Solution Digestion applications. For the Peptide Cleanup labware requirements, see "Labware" on page 360. For the In-Solution Digestion: Multi-Plate labware requirements, see "In-Solution Digestion: Multi-Plate v2.0 User Guide" on page 235.

Labware	Part number
1.2 mL Deep-Well PolyPro Clear Plates (qty 2)	ABgene AB-1127
96-Well U-Bottom PolyPro White Plates (qty 11)	Greiner 650207
250-µL Pipette Tips (qty 1)	Agilent 19477-02
12-Column Low-Profile Reservoirs (qty 4)	Agilent 201280-100
96-Well Round-Bottom, Clear Plates (qty 2)	Greiner 650201
96-Well PCR Plates (qty 3)	Eppendorf 30129300

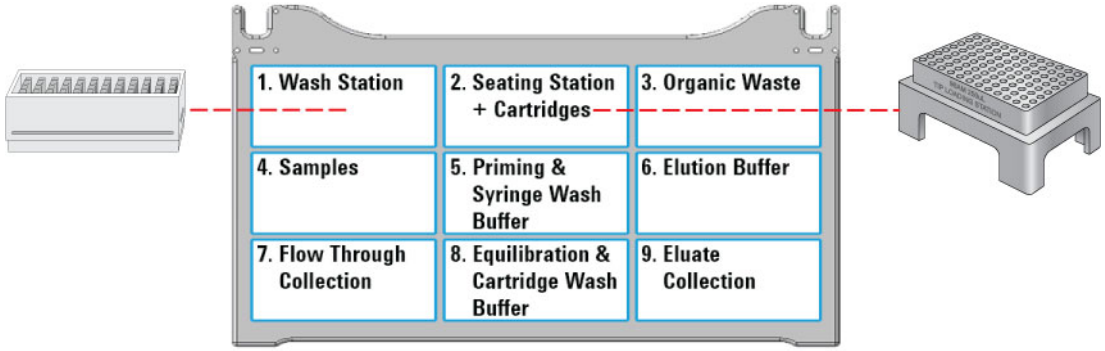
Labware

Labware requirements vary depending on experimental design. The following table provides a complete list labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3, 5–8
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3, 5–8
96 Agilent, 2mL Square Deep Well labware	Agilent 204353-100	3, 7
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—8
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7, 9**
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3—9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	4, 7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	5, 6, 8

*For dimensionally equivalent alternatives and other labware details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner PCR plate is not compatible with the 25 µL cartridges at deck locations 7 and 9.

Reagents

The volume, type, and concentration of reagents required for peptide cleanup vary depending on sample characteristics and the desired analytical result. Consult published literature for reagent recommendations for sample and surface chemistry combinations. See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Peptide Cleanup application.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 361.

Preparing the solutions

The following solutions are required for the Peptide Cleanup protocol:

- Priming & Syringe Wash Buffer
- Equilibration & Cartridge Wash Buffer
- Elution Buffer

CAUTION

A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Peptide Cleanup

The Reagent Volume Calculator is a Microsoft Excel file that contains the following:

- *Calculator worksheet*. You enter the number of columns to process, whether to perform the Collect Flow Through option, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

  Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

- *Reagent Recipe worksheet*. You enter the concentrations of each component in your reagent, and the worksheet calculates the recipe volumes required.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figures show the worksheets of the Reagent Volume Calculator.

Figure Peptide Cleanup Reagent Volume Calculator worksheet

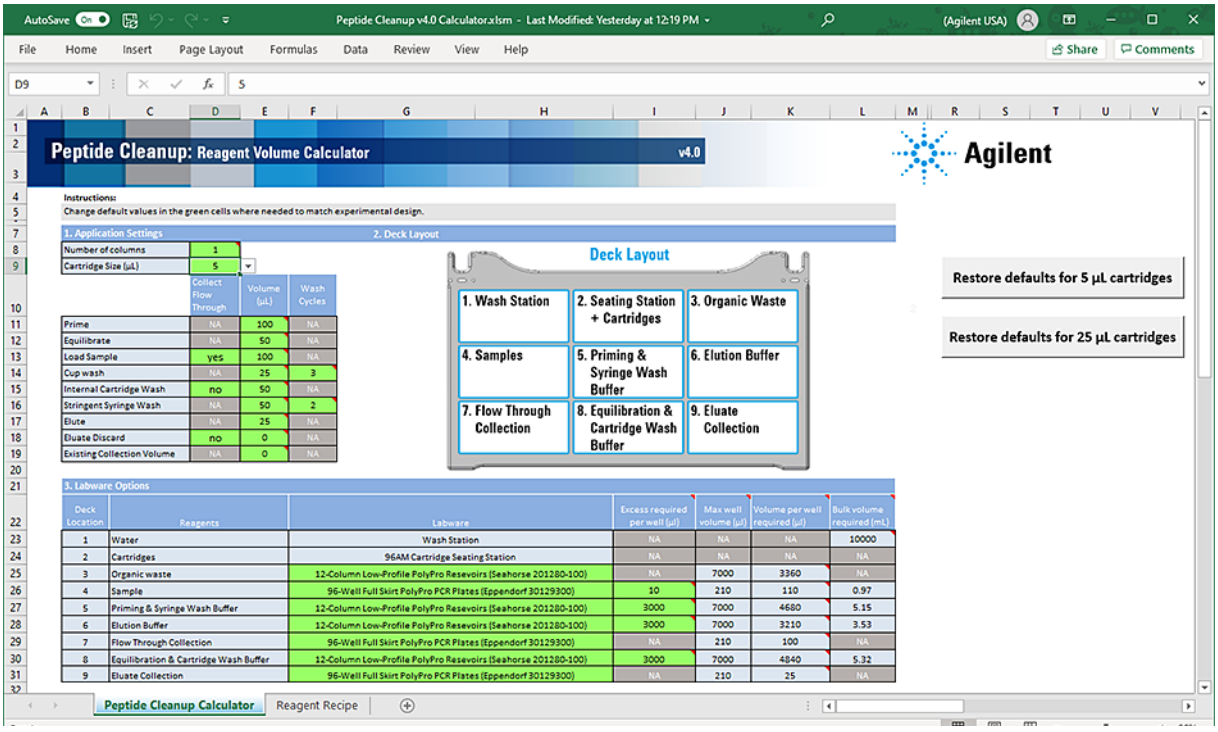
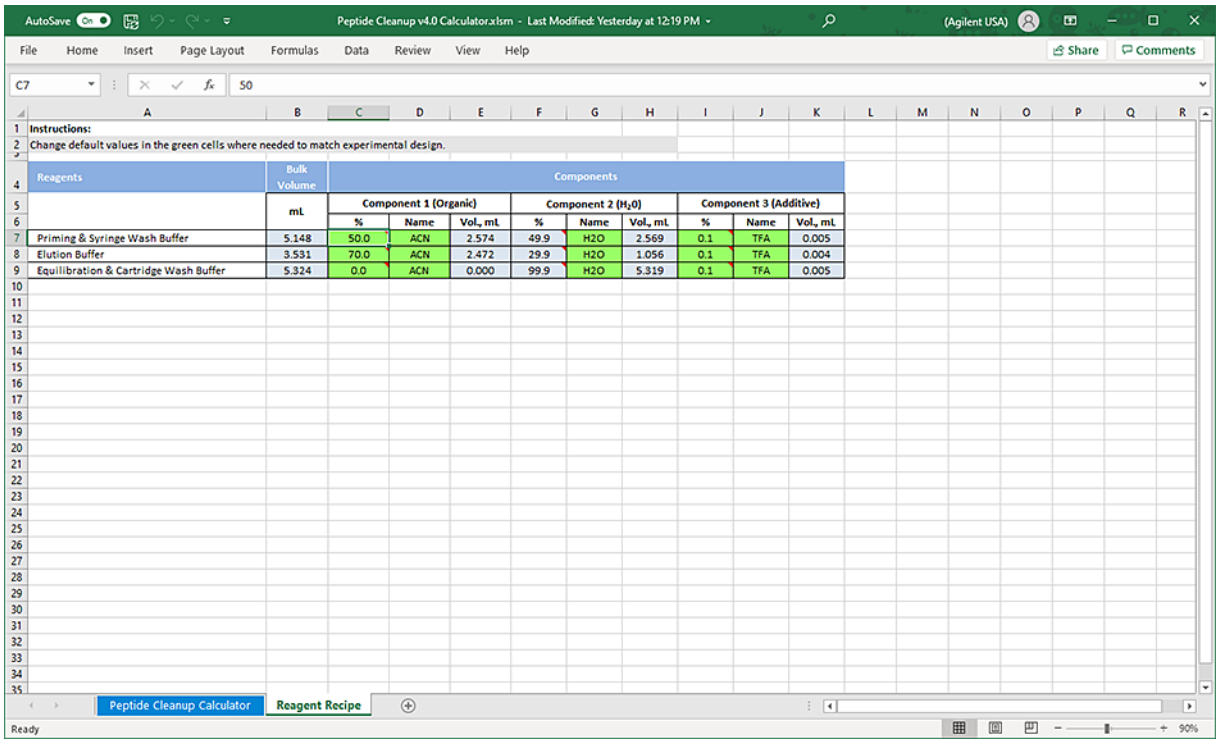


Figure Peptide Cleanup Reagent Recipe worksheet



Instructions:												
Change default values in the green cells where needed to match experimental design.												
Reagents	Bulk Volume	Components										
	mL	Component 1 (Organic)			Component 2 (H_2O)			Component 3 (Additive)				
		%	Name	Vol., mL	%	Name	Vol., mL	%	Name	Vol., mL		
Priming & Syringe Wash Buffer	5.148	50.0	ACN	2.574	49.9	H2O	2.569	0.1	TFA	0.005		
Elution Buffer	3.531	70.0	ACN	2.472	29.9	H2O	1.056	0.1	TFA	0.004		
Equilibration & Cartridge Wash Buffer	5.324	0.0	ACN	0.000	99.9	H2O	5.319	0.1	TFA	0.005		

Preparing the buffers

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the AssayMAP Peptide Cleanup chemistry.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming & Syringe Wash Buffer (deck location 5)	Typically 50% or greater organic solution (must be >25% organic), acidic, and identical in composition to the Elution Buffer to simplify the solution preparation, for example, 70% ACN: 29.9% H_2O : 0.1% TFA The high-percentage organic composition is essential for reversed-phase cartridges as they ship dry and must be wetted with a high percentage organic solution or they will have very little binding capacity. This solution, the other solutions used in this application, and the sample are typically acidic. Other more mass-spec-friendly acids can substitute for TFA.

Reagent (deck location)	Composition and comments
Elution Buffer (deck location 6)	High percentage organic and acidic solution, for example, 70% ACN: 29.9% H_2O : 0.1% TFA
Equilibration & Cartridge Wash Buffer (deck location 8)	Very low or no organic and acidic solution. Typically, similar in the percentage organic and acidity to the sample, for example, 99.9% H_2O : 0.1% TFA

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and cartridge positions on the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 364.

To dispense the solutions into the labware:

1. *Optional*. Label each piece of labware so that you can easily identify them.
2. Add the specified volume of the Priming Buffer & Syringe Wash Buffer into the labware to be placed at deck location 5.
3. Add the specified volume of Elution Buffer into the labware to be placed at deck location 6.
4. Add the specified volume of Equilibration & Cartridge Wash Buffer into the labware to be placed at deck location 8.
5. If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT To minimize evaporation, prepare the samples immediately before running the Peptide Cleanup protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to the optimal binding conditions (for example, low pH and low organic).
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the buffer composition

One of the most important considerations for highly efficient and unbiased binding of peptides to the reversed-phase resin is the pH of the sample, which should be acidic for standard, low-pH peptide cleanup with C18 or RP-S cartridges or basic with RP-S cartridges.

Peptides are amphoteric molecules with a diverse range of physiochemical properties. For some classes of peptides, cleanup under high-pH conditions may help to promote retention (e.g., highly basic peptides), or preserve acid-labile modifications (e.g., histidine phosphorylation). You can acidify samples for low-pH peptide cleanup before loading onto AssayMAP C18 and RP-S cartridges generally by adding TFA, formic acid, or acetic acid. Sample pH for high-pH peptide cleanup is commonly adjusted using aqueous solutions of ammonium formate or ammonium acetate titrated to a pH > 10 using ammonium hydroxide. You can use the Reagent Transfer utility to perform these pH adjustments. For instructions, see "Reagent Transfer v3.0 User Guide" on page 525.

Samples containing organic solvents or some types of detergents should be avoided as they might bias the peptides that bind the column. For example, loading samples in a buffer containing greater than 5% acetonitrile will inhibit binding of hydrophilic peptides. Silica-based C18 cartridges are at risk with a sample pH higher than 8. If you have concerns about a specific buffer component, you should examine scientific literature for the known effects of this type of molecule on reversed-phase resins. You can use the Reagent Transfer utility to modify the composition of your samples with a dilution or pH adjustment, especially after using the In-Solution Digestion workflow.

Determining the volume of sample to load

The AssayMAP Peptide Cleanup protocol permits loading up to 1000 µL of sample onto AssayMAP cartridges. For sample volumes > 250 µL, the protocol will iteratively load samples onto cartridges to stay within the maximum syringe volume (250 µL) of the Bravo 96AM Head.

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of peptide that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through.

  For a single species of peptide, the quantitative binding capacity is relatively straightforward. The quantitative binding capacity for a mixture of peptides is more complex due to the differences in relative hydrophobicity of the peptides, which results in competitive binding in situations where the ratio of binding sites to mass loaded is low.

  Examples of how sample mass loaded and the relative hydrophobicity of peptides can affect recovery in a complex peptide mixture, see Agilent app note 5991-2957EN in the "Reference library" on page 392. To avoid or minimize sample bias during peptide cleanup, it is critical to load sample masses that are less than the mass at which hydrophillic peptides are lost.
- *Total binding capacity*. The maximum mass of peptide that can bind to the cartridge. This can only be achieved by loading significantly more peptide than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity and will result in the loss of hydrophillic peptides.

See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed information about the binding capacity for the 5 and 25 µL RPS and C18 cartridges.

What is the concentration of the target in the sample?

If you know the approximate concentration of the target molecule in your sample and you are working within the quantitative binding range of the cartridge, you can determine the volume of sample to load as follows:

$$\text{µL sample to load} = \frac{\text{µg peptide desired}}{\text{µg/µL of peptide in sample}}$$

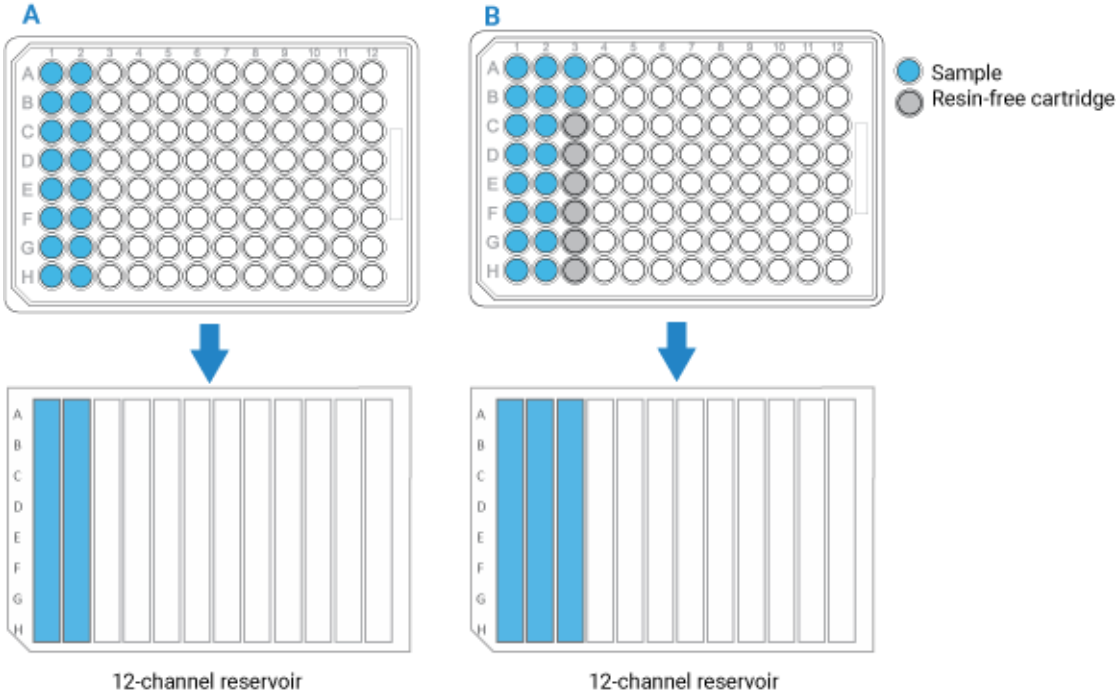
Preparing the sample plates

Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.
- The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the Bravo Platform applies differential pressure to seat cartridges based upon the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use the AssayMAP Resin-Free cartridges (Agilent part number G5496-60009) to fill the empty column and row positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash step.

Figure Example of sample microplate and reservoir layout: A) Multiple of 8 samples, B) Not a multiple of 8



See "Labware" on page 360 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION

A small volume excess is required in all labware types to ensure proper volume transfer.

An excess (overage) volume ensures that a microplate well or column does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide*, which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Peptide Cleanup protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the peptides from the cartridges.

For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the cartridge seating station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 361.
- Prepare the samples. See "Preparing the samples" on page 364.

- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup protocol to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the 96AM Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

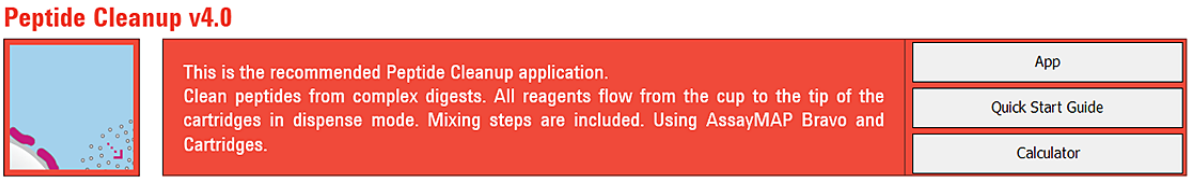
IMPORTANT Do not allow wetted cartridges to dry out. Agilent Technologies does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 358.

Setting up the protocol

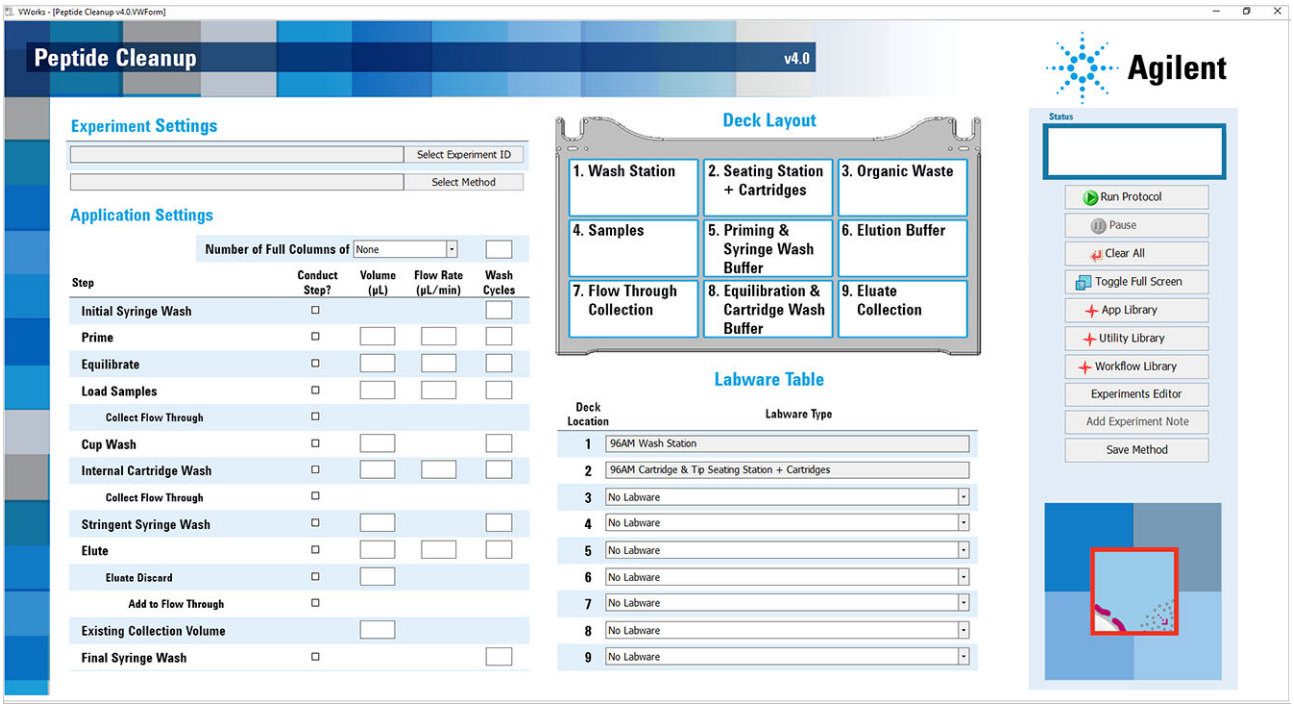
Before starting the protocol, make sure the appropriate selections and values are specified in the Peptide Cleanup application.

To set up the protocol:

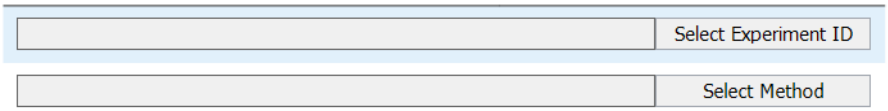
1. Open the **App Library**.
2. Locate **Peptide Cleanup**, and then click **App**.



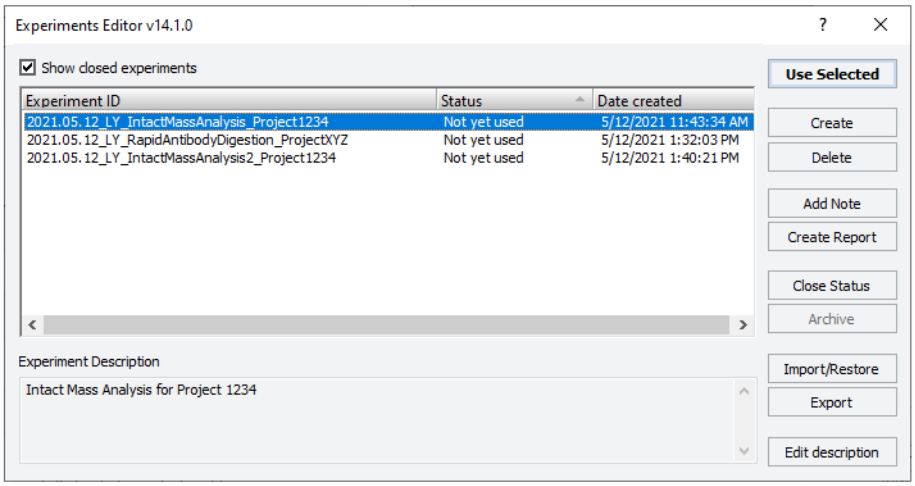
The Peptide Cleanup application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 374.
- To modify the method, proceed to step 6.

VWorks Plus. Only VWorks administrators or technicians may modify and save methods.

6 In the **Application Settings** area, specify the cartridge settings:

a Select the cartridge size from the list:
- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION **If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.**

CAUTION **If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.**

IMPORTANT Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 376.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 5) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10
Equilibrate	Aspirates Equilibration Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates samples (deck location 4) into the syringes, and then dispenses them through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 7).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–1000	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the sample flow-through in the Organic Waste plate (deck location 3).	–	–	–	–
Cup Wash	Rinses the cartridge cups with Cartridge Wash Buffer (deck location 8), and then discards the liquid into the Organic Waste plate (deck location 3).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Internal Cartridge Wash	Aspirates Cartridge Wash Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 7).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the Internal Cartridge Wash flow-through in the Organic Waste plate (deck location 3).	–	–	–	–
Stringent Syringe Wash	Aspirates Syringe Wash Buffer (deck location 5) into the syringes, and then dispenses it into the Organic Waste plate (deck location 3).	5 µL:	50	–	2
		25 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates Elution Buffer (deck location 6) into the syringes, and then dispenses it through the cartridges into the Eluate Collection plate (deck location 9).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0–250	0.1–500	0–10
Eluate Discard	If selected, a specified initial volume of the eluate is discarded into the Organic Waste plate (deck location 3), or collected in the Flow Through Collection plate (deck location 7).	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–250	–	–
Add to Flow Through	If selected, collects the Eluate Discard in the Flow Through Collection plate (deck location 7). If not selected, discards the Eluate Discard into the Organic Waste plate (deck location 3).	–	–	–	–
Existing Collection Volume	Specifies the volume of liquid present in the Eluate Collection plate (deck location 9) at the beginning of the run.	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–300	–	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

**For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 377.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 388.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT The protocol will display an error messages if cartridges are missing.

Starting the protocol run

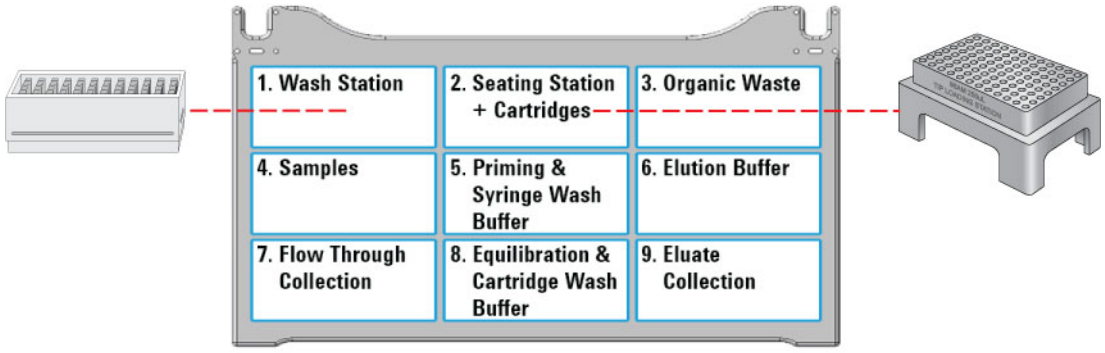
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

Note: The Greiner PCR plates are not compatible with the 25 µL cartridges at deck locations 7 and 9.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

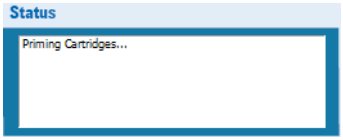
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

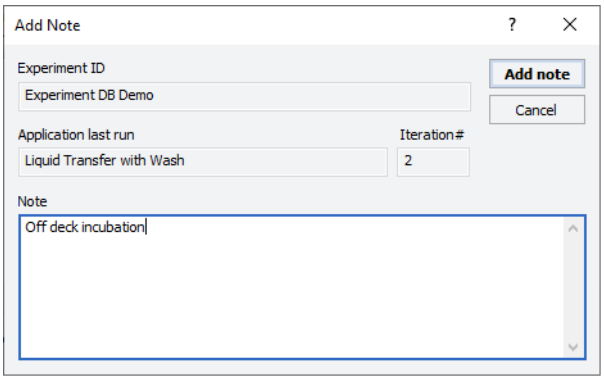
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



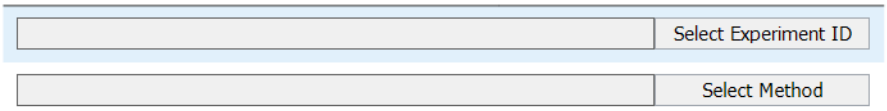
2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

1. Remove used labware from the deck.
2. Discard leftover reagents appropriately.
3. *Optional*. Conduct stringent washing of the syringes:
   a. Open the **Syringe Wash** utility  .
   b. If applicable, click **Select Experiment ID** to open the Experiments Editor.



   c. In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.
   d. Click **Select Method** to select and load the method for this utility.
   e. Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.
   f. Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Peptide Cleanup protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used,* the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used,* the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the packed resin bed and properly wets the surface of the resin. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP reversed-phase cartridges (C18, RPS, and RPW) used with the Peptide Cleanup application require a Priming Buffer containing at least 25% organic solvent. As illustrated in Agilent app note 5991-6478EN ("Reference library" on page 392), reversed-phase cartridges (C18, RPS, and RPW) that are primed with solutions containing a low percentage of organic solvent have greatly reduced binding capacity. Agilent recommends using a priming solution that contains at least 50% of an organic solvent such as acetonitrile. This step is selected by default. **Volume (µL)**. The default volume is sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridges: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow Rate (µL/min):** A flow rate lower than the default value diminishes the ability to effectively remove entrained air from the cartridge. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–500 **Wash Cycles:** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the Organic Waste plate The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP reversed-phase cartridges (C18, RPS, and RPW) typically used with the Peptide Cleanup application require an equilibration solution that has a very low concentration of or no organic solvent for effective binding during the sample loading step. This step is selected by default. **Volume (µL)**. The default volume is equal to 10-column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow Rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min using the default volume may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash Cycles:** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the sample loading begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates sample into the syringes, and then performs an external syringe wash at the wash station to remove any sample remaining on the outside of the probes before mounting the cartridges. The samples are dispensed through the cartridges into the Flow Through Collection plate or Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any sample on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. The protocol accommodates sample volumes up to 1000 µL to be dispensed through AssayMAP peptide cleanup cartridges. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. Each syringe has a maximum capacity of 250 µL. When sample volumes are greater than 250 µL, the protocol will iteratively load samples onto cartridges. To determine the number and the volume of iterative loads, the protocol uses the following formulas: • *# of times to load* = Total sample volume/250, and the result is rounded up to nearest integer • *Volume of each load* = Sample volume/# of times to load For example, if the total sample volume is 900 µL, then: *# times to load* = 900/250 = 3.6, which is rounded up to 4 *Volume of each load* = 900/4 = 225 If Collect Flow Through is selected for the Load Samples step, be sure that the Flow Through Collection plate has sufficient maximum well capacity. For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. **IMPORTANT** Be sure to include the recommended labware-specific volume overage to prevent air from entering the cartridge. For more information, see "Transferring the samples to the microplate" on page 367. To determine the volume of sample to load, see "Determining the volume of sample to load" on page 365. This step is selected by default.

Protocol step	Guidelines and notes
	Volume (µL). The volume of sample should be balanced with the sample concentration and the mass capacity of the cartridge. Large sample volumes (> 250 µL) may require slightly more excess sample due to evaporation. • Default: 100 • Practical: 10–1000 • Range: 0–1000 *Note:* The lower the sample volume the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min)**. The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than the default may not significantly increase analyte binding, and using flow rates faster than the default will decrease the quantitative binding capacity of the cartridges. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash Cycles.** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed in the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step is dispensed in the Organic Waste plate. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is selected by default.

Protocol step	Guidelines and notes
Cup Wash	This step removes the residual sample liquid that may remain above the resin bed after the Load Samples step. The Cup Wash step aspirates Cartridge Wash Buffer into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the Organic Waste plate, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL):** Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash Cycles:** Each wash cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash	This step uses Cartridge Wash Buffer to wash non-specifically bound molecules from the resin bed. In preparation for the Internal Cartridge Wash, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Cartridge Wash Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates the Cartridge Wash Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station If the Load Samples step is selected, the first 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) of Cartridge Wash Buffer is dispensed as a sample chase at the Load Samples flow rate. Next, the Internal Cartridge Wash volume minus the chase volume is dispensed at the Internal Cartridge Wash flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. This step is selected by default. **Volume (µL).** Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow Rate (µL/min):** A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash Cycles.** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Collect Flow Through	If this step is selected, the flow-through from the Internal Cartridge Wash step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from the Internal Cartridge Wash step is dispensed into the Organic Waste plate. This step is not selected by default.
Stringent Syringe Wash	This step cleans the syringes with the Syringe Wash Buffer prior to elution. The Stringent Syringe Wash step aspirates the Syringe Wash Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the Organic Waste plate. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL).** Volumes higher than the default volume are unlikely to improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash Cycles.** A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Elute	This step uses Elution Buffer to elute bound peptides from the cartridges. In preparation for elution, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Elution Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges, and then the cartridges are parked at the seating station. After the elution, the samples are mixed in the Eluate Collection plate and the syringes are washed at the wash station. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples will not be mixed. You can also select the Eluate Discard and Add to Flow Through substeps, which are described in the following rows of this table. This step is selected by default. **Volume (µL):** The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges, or 50–75 µL for 25 µL cartridges). The default volumes are conservative and significantly higher than the minimum expected with a strong Elution Buffer. *Note:* The Eluate Collection plate must be able to accommodate the total volume, which is determined by summing the net elution volume (Elute volume - Eluate Discard volume) and the Existing Collection Volume. For labware-specific maximum well volumes, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. • Volume for 5 µL cartridges: – Default: 25 – Practical: 10–30 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 125 – Practical: 50–150 – Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing.

Protocol step	Guidelines and notes
Elute (continued)	**Flow Rate (µL/min):** A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more buffer may be required to get the same elution yield at high flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500 **Wash Cycles.** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 Range: 0–10
Eluate Discard	This substep of the Elute step permits a specified volume of the eluate from the cartridges to be discarded before the eluate starts to be collected during the Elute step. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the Elution Buffer at the Elute flow rate through the cartridges. If the Eluate Discard step is selected, the specified volume is dispensed into the Organic Waste plate or Flow Through Collection plate (if the Add to Flow Through step is selected). The remaining Elution Buffer is dispensed through cartridges at the Elute flow rate into the Eluate Collection plate. **Example**: If the Elute, Eluate Discard, and Add to Flow Through steps are all selected with the following settings: Elute volume = 15 µL (5 µL cartridges) or 40 µL (25 µL cartridges) Eluate Discard volume = 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) the first 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) eluate from the cartridges will be discarded into the Flow Through Collection plate, and the remaining 13 µL (5 µL cartridges) or 30 µL (25 µL cartridges) eluate will be collected in the Eluate Collection plate. Select the Eluate Discard step in situations where minimizing the volume of eluate is important. For AssayMAP cartridges, the initial elution volume (~2 µL for 5 µL cartridges or ~10 µL for the 25 µL cartridges) contains small or no measurable amounts of analyte. This step is not selected by default. **Volume (µL).** The first volume of eluate that will be discarded during the Elute step. This value can equal, but cannot exceed the Elute volume. • Default: 0 • Practical: – 5 µL cartridges: 0–2 – 25 µL cartridges: 0–10 • Range: 0–250

Protocol step	Guidelines and notes
Add to Flow Through	If selected, this step dispenses the Eluate Discard volume into the Flow Through Collection plate. If the Add to Flow Through step is not selected, the Eluate Discard is dispensed into the Organic Waste plate. This step is not selected by default. *Note:* The Add to Flow Through step is an option only if both the Elute and Eluate Discard steps have been selected.
Existing Collection Volume	This step enables you to specify an amount of liquid that is in the wells of the Eluate Collection plate at the beginning of the run. The Existing Collection Volume and the net volume from the Elute step (Elute volume - Eluate Discard volume) feeds into logic that adjusts the well-bottom offset for sample elution, calculates the eluate mixing volume, and dynamically moves the head into and out of the wells during elution and eluate mixing in a volume-dependent manner. For the maximum practical working volumes of the labware for the Eluate Collection plate, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. Select this step when the Eluate Collection plate contains a volume of liquid useful for immediately diluting the eluates, for adjusting the pH of the eluates, or to aid in the recovery of small volumes of eluates from AssayMAP cartridges. **Volume (µL):** • Default: 0 • Practical: 0—250 • Range: 0—300
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, the 10 µL of elution buffer will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL of DI water into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. *Note:* In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Peptide Cleanup protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	2	Parks all cartridges that might have been loaded on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes.
Prime	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	5	Aspirates 10 µL of Priming Buffer for the cartridge air-gap prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	5	Aspirates the Priming Buffer.
	2	Mounts the cartridges onto the head.
	3	Dispenses the Priming Buffer through the cartridges and into Organic Waste plate to prime the cartridges.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	8	Aspirates 10 µL of Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Equilibration Buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Equilibration Buffer through the cartridges to equilibrate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Load Samples	4	Aspirates the samples into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	7	Dispenses the samples through the cartridges and into the Flow Through Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash	8	Aspirates the Cartridge Wash Buffer.
	2	Washes the cartridge cups and exercises the cartridges off task.
	3	Dispenses the Cartridge Wash Buffer into the Organic Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	8	Aspirates 10 µL of Cartridge Wash Buffer for the cartridge air-gap prevention step.
	2	Dispenses the remaining 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Cartridge Wash Buffer into the syringes for sample chase and the Internal Cartridge Wash steps.
	2	Mounts the cartridges on the head.
	3	Dispenses 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) Cartridge Wash Buffer through the cartridges at the Load Samples flow rate for the sample chase step.
	3	Dispenses the remaining Cartridge Wash buffer through the cartridges at the Internal Cartridge Wash flow rate and into the Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Stringent Syringe Wash	5	Aspirates the Syringe Wash Buffer.
	3	Dispenses the Syringe Wash Buffer into the Organic Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Elute	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	6	Aspirates 10 µL of Elution Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Elution Buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates the Elution Buffer.
	2	Mounts the cartridges on the head.
	9	Elutes the samples into the Eluate Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	9	Mixes the eluates.
	1	Washes the syringes.
Final Syringe Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the syringes.

Reference library

1 Russel, J., Van Den Heuvel, Z., Bovee, M. & Murphy, S., Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-2957EN, 2016

2 Bovee, M., Krahenbuhl, A. & Murphy, S., Rapid Antibody Digestion Enabled by Automated Reversed-Phase Desalting on the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-6478EN, 2016

See the Agilent AssayMAP Bravo Citation Index for published papers that used the AssayMAP Bravo Platform.

12 Peptide Cleanup: Aspiration Mode v3.0 User Guide

This chapter contains the following topics:

Note: This section presents instructions for using the Peptide Cleanup: Aspiration Mode application. If you are using the Dispense Mode version, see "Peptide Cleanup v4.0 User Guide" on page 355.

App description

Peptide Cleanup: Aspiration Mode v3.0. This application enables automated cleanup of from 1 to 96 peptide samples in a single run. This application aspirates the sample and wash solutions up through the cartridge resin bed rather than dispensing them through the resin bed, which is how the standard Peptide Cleanup application functions.

For most customers, Agilent recommends using the standard Peptide Cleanup application instead of the Peptide Cleanup: Aspiration Mode application. Although the two applications yield similar results, the standard Peptide Cleanup application is less sensitive to clogging and, therefore, more robust. However, some customers find the Peptide Cleanup: Aspiration Mode application provides slightly better purification.

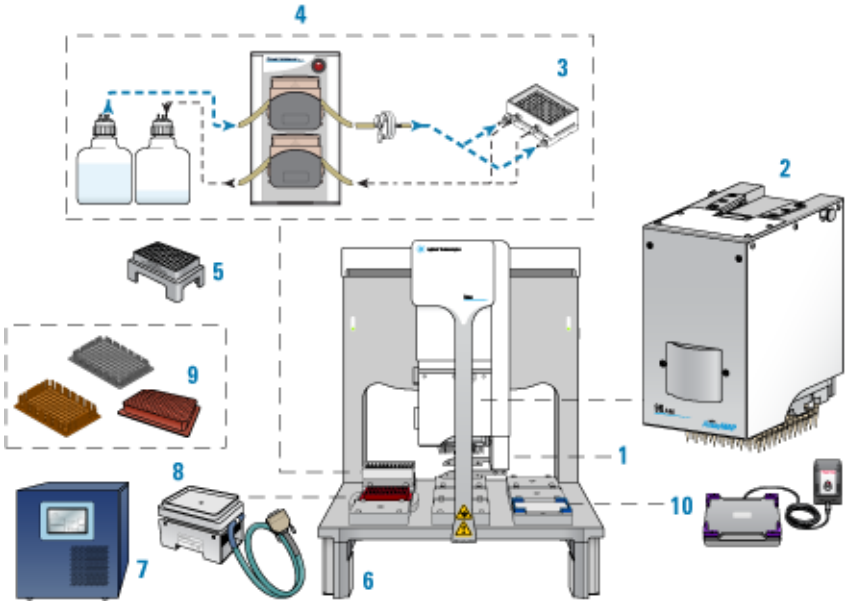
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Peptide Cleanup: Aspiration Mode protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION

To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Note: The 25-µL have not yet been optimized on the Peptide Cleanup: Aspiration Mode application. If you are interested in trying them on this application, contact Agilent Customer Service for advice.

Optional equipment. You might need the following when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate vacuum concentrator for concentration and drying of samples
- Microplate sealer, such as the PlateLoc Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Peptide Cleanup on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Note: This application has not yet been optimized for the 25 µL cartridges.

Cartridge type	Part number
AssayMAP Reversed-Phase (C18) cartridge rack	5190-6532
AssayMAP Reversed-Phase (RP-S) cartridge rack	G5496-60033
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or to fill remaining positions in columns that are partially occupied by the C18 and RP-S cartridges. For details, see "Preparing the samples" on page 402.	G5496-60009

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.

Starter kits

The following table lists the two starter kits that are available. Each starter kit contains both cartridges and labware.

Starter Kit	Part number
AssayMAP Digestion and RP-S Cleanup Starter Kit— Contains 96 Reversed-Phase (RP-S) cartridges and Labware for In-Solution Digestion: Multi-Plate and Peptide Cleanup.	G5496-60034
AssayMAP Digestion and C18 Cleanup Starter Kit— Contains 96 Reversed-Phase (C18) cartridges and Labware for In-Solution Digestion: Multi-Plate and Peptide Cleanup.	G5496-60013

The following table lists labware that are included in the Peptide Cleanup Starter Kit.

Note: The labware included in the starter kits are for both the Peptide Cleanup: Aspiration Mode and the In-Solution Digestion applications. For the Peptide Cleanup: Aspiration Mode labware requirements, see "Labware" on page 398. For the In-Solution Digestion: Multi-Plate labware requirements, see "In-Solution Digestion: Multi-Plate v2.0 User Guide" on page 235.

Labware	Part number
1.2 mL Deep-Well PolyPro Clear Plates (qty 2)	ABgene AB-1127
96-Well U-Bottom PolyPro White Plates (qty 11)	Greiner 650207
250-µL Pipette Tips (qty 1)	Agilent 19477-02
12-Column Low-Profile Reservoirs (qty 4)	Agilent 201280-100

Labware	Part number
96-Well Round-Bottom, Clear Plates (qty 2)	Greiner 650201
96-Well PCR Plates (qty 3)	Eppendorf 30129300

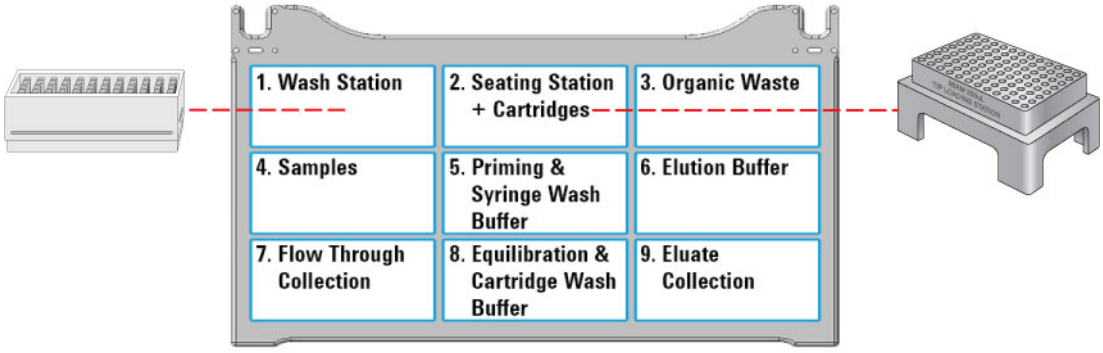
Labware

Labware requirements vary depending on experimental design. The following table provides a complete list labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3, 5–8
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	5–8
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—8
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7, 9
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	4, 7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	5–8

*For dimensionally equivalent alternatives and other labware details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Reagents

The volume, type, and concentration of reagents required for peptide cleanup vary depending on sample characteristics and the desired analytical result. Consult published literature for reagent recommendations for sample and surface chemistry combinations. See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Peptide Cleanup application.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 399.

Preparing the solutions

The following solutions are required for the Peptide Cleanup: Aspiration Mode protocol:

- Priming & Syringe Wash Buffer
- Equilibration & Cartridge Wash Buffer
- Elution Buffer

CAUTION

A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Peptide Cleanup: Aspiration Mode

The Reagent Volume Calculator is a Microsoft Excel file that contains a Calculator worksheet. You enter the number of columns to process, whether to perform the Collect Flow Through options, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

To use the Reagent Volume Calculator:

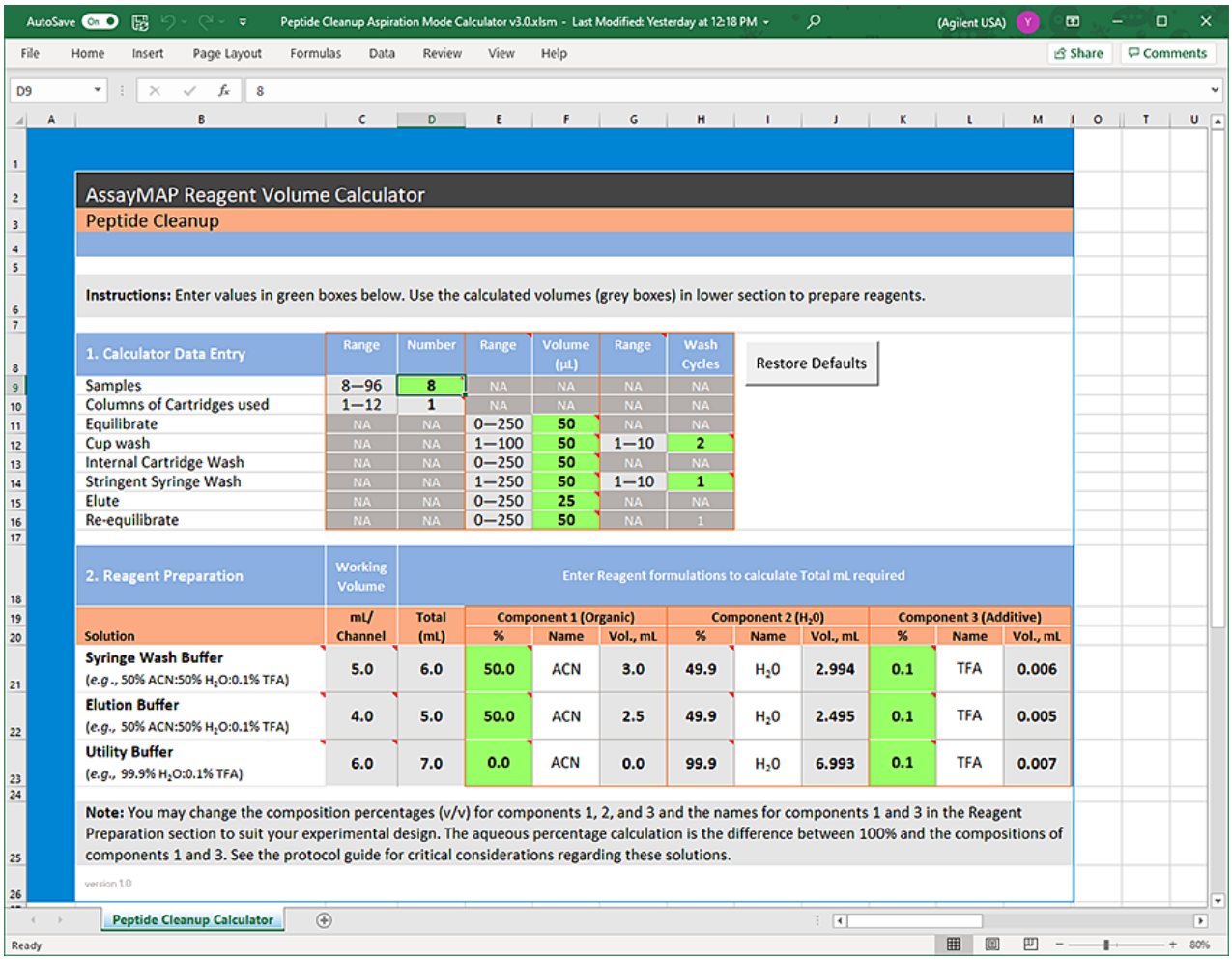
1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click **Restore Defaults**.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figure shows the worksheet of the Reagent Volume Calculator.

Figure Reagent Volume Calculator for Peptide Cleanup: Aspiration Mode



Preparing the buffers

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the AssayMAP Peptide Cleanup: Aspiration Mode chemistry.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming & Syringe Wash Buffer (deck location 5)	Typically 50% or greater organic solution (must be >25% organic), acidic, and identical in composition to the Elution Buffer to simplify the solution preparation, for example, 70% ACN: 29.9% H_2O : 0.1% TFA The high-percentage organic composition is essential for reversed-phase cartridges as they ship dry and must be wetted with a high percentage organic solution or they will have very little binding capacity. This solution, the other solutions used in this application, and the sample are typically acidic. Other more mass-spec-friendly acids can substitute for TFA.
Elution Buffer (deck location 6)	High percentage organic and acidic solution, for example, 70% ACN: 29.9% H_2O : 0.1% TFA
Equilibration & Cartridge Wash Buffer (deck location 8)	Very low or no organic and acidic solution. Typically, similar in the percentage organic and acidity to the sample, for example, 99.9% H_2O : 0.1% TFA

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and cartridge positions on the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 402.

To dispense the solutions into the labware:

1. *Optional*. Label each piece of labware so that you can easily identify them.
2. Add the specified volume of the Priming Buffer & Syringe Wash Buffer into the labware to be placed at deck location 5.

3 Add the specified volume of Elution Buffer into the labware to be placed at deck location 6.

4 Add the specified volume of Equilibration & Cartridge Wash Buffer into the labware to be placed at deck location 8.

5 If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT

To minimize evaporation, prepare the samples immediately before running the Peptide Cleanup: Aspiration Mode protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to the optimal binding conditions (for example, low pH and low organic).
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the buffer composition

One of the most important considerations for highly efficient and unbiased binding of peptides to the reversed-phase resin is the pH of the sample, which should be acidic for standard, low-pH peptide cleanup with C18 or RP-S cartridges or basic with RP-S cartridges.

Peptides are amphoteric molecules with a diverse range of physiochemical properties. For some classes of peptides, cleanup under high-pH conditions may help to promote retention (e.g., highly basic peptides), or preserve acid-labile modifications (e.g., histidine phosphorylation). You can acidify samples for low-pH peptide cleanup before loading onto AssayMAP C18 and RP-S cartridges generally by adding TFA, formic acid, or acetic acid. Sample pH for high-pH peptide cleanup is commonly adjusted using

aqueous solutions of ammonium formate or ammonium acetate titrated to a pH > 10 using ammonium hydroxide. You can use the Reagent Transfer utility to perform these pH adjustments. For instructions, see "Reagent Transfer v3.0 User Guide" on page 525.

Samples containing organic solvents or some types of detergents should be avoided as they might bias the peptides that bind the column. For example, loading samples in a buffer containing greater than 5% acetonitrile will inhibit binding of hydrophilic peptides. Silica-based C18 cartridges are at risk with a sample pH higher than 8. If you have concerns about a specific buffer component, you should examine scientific literature for the known effects of this type of molecule on reversed-phase resins. You can use the Reagent Transfer utility to modify the composition of your samples with a dilution or pH adjustment, especially after using the In-Solution Digestion workflow.

Determining the volume of sample to load

The AssayMAP Peptide Cleanup protocol permits loading up to 1000 µL of sample onto AssayMAP cartridges. For sample volumes > 250 µL, the protocol will iteratively load samples onto cartridges to stay within the maximum syringe volume (250 µL) of the Bravo 96AM Head.

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of peptide that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through.

  For a single species of peptide, the quantitative binding capacity is relatively straightforward. The quantitative binding capacity for a mixture of peptides is more complex due to the differences in relative hydrophobicity of the peptides, which results in competitive binding in situations where the ratio of binding sites to mass loaded is low.

  Examples of how sample mass loaded and the relative hydrophobicity of peptides can affect recovery in a complex peptide mixture, see Agilent app note 5991-2957EN in the "Reference library" on page 423. To avoid or minimize sample bias during peptide cleanup, it is critical to load sample masses that are less than the mass at which hydrophillic peptides are lost.
- *Total binding capacity*. The maximum mass of peptide that can bind to the cartridge. This can only be achieved by loading significantly more peptide than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity and will result in the loss of hydrophillic peptides.

See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed information about the binding capacity for the RPS and C18 cartridges.

What is the concentration of the target in the sample?

If you know the approximate concentration of the target molecule in your sample and you are working within the quantitative binding range of the cartridge, you can determine the volume of sample to load as follows:

$$\text{µL sample to load} = \frac{\text{µg peptide desired}}{\text{µg/µL of peptide in sample}}$$

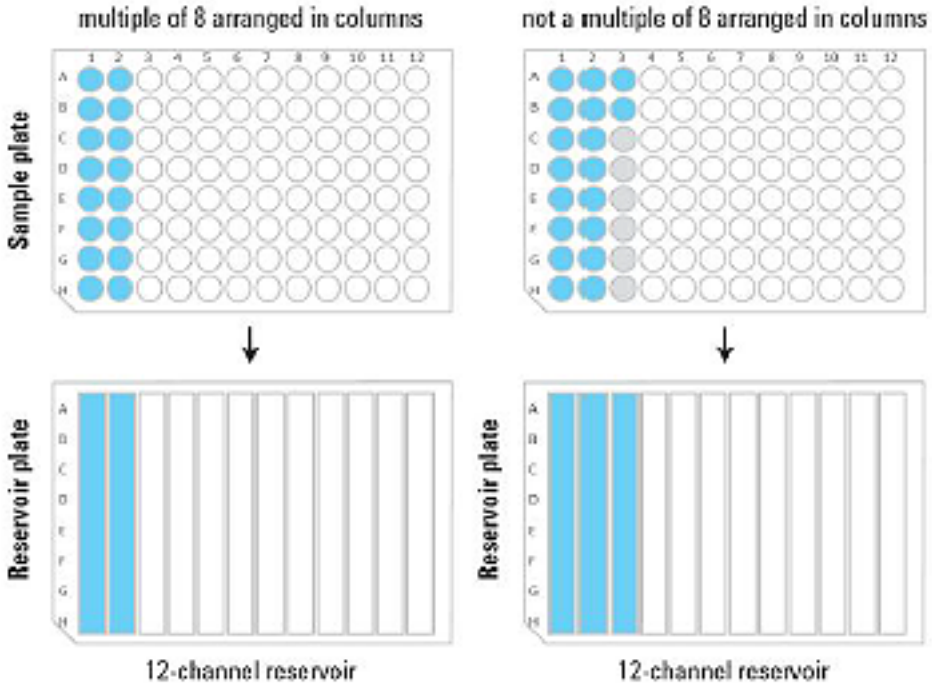
Preparing the sample plates

Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.
- The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the Bravo Platform applies differential pressure to seat cartridges based upon the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use the AssayMAP Resin-Free cartridges (Agilent part number G5496-60009) to fill the empty column and row positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash step.

Figure Example of sample microplate and reservoir layout



See "Labware" on page 398 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

An excess volume (overage) ensures that the sample does not fully deplete. Aspirating from depleted wells can cause air to enter the cartridges, thus compromising results. Modify this excess volume accordingly if evaporation of volatile solvents is a concern.

See the *Labware Reference Guide* for labware-specific overage recommendations. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility to transfer the samples. For instructions, see "Reagent Transfer v3.0 User Guide" on page 525.
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Peptide Cleanup: Aspirate Mode protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the peptides from the cartridges.

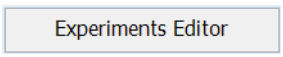
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the cartridge seating station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 399.
- Prepare the samples. See "Preparing the samples" on page 402.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup protocol to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the 96AM Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

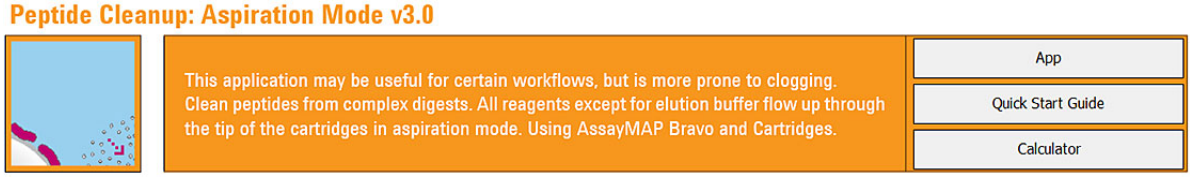
IMPORTANT Do not allow wetted cartridges to dry out. Agilent does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 396.

Setting up the protocol

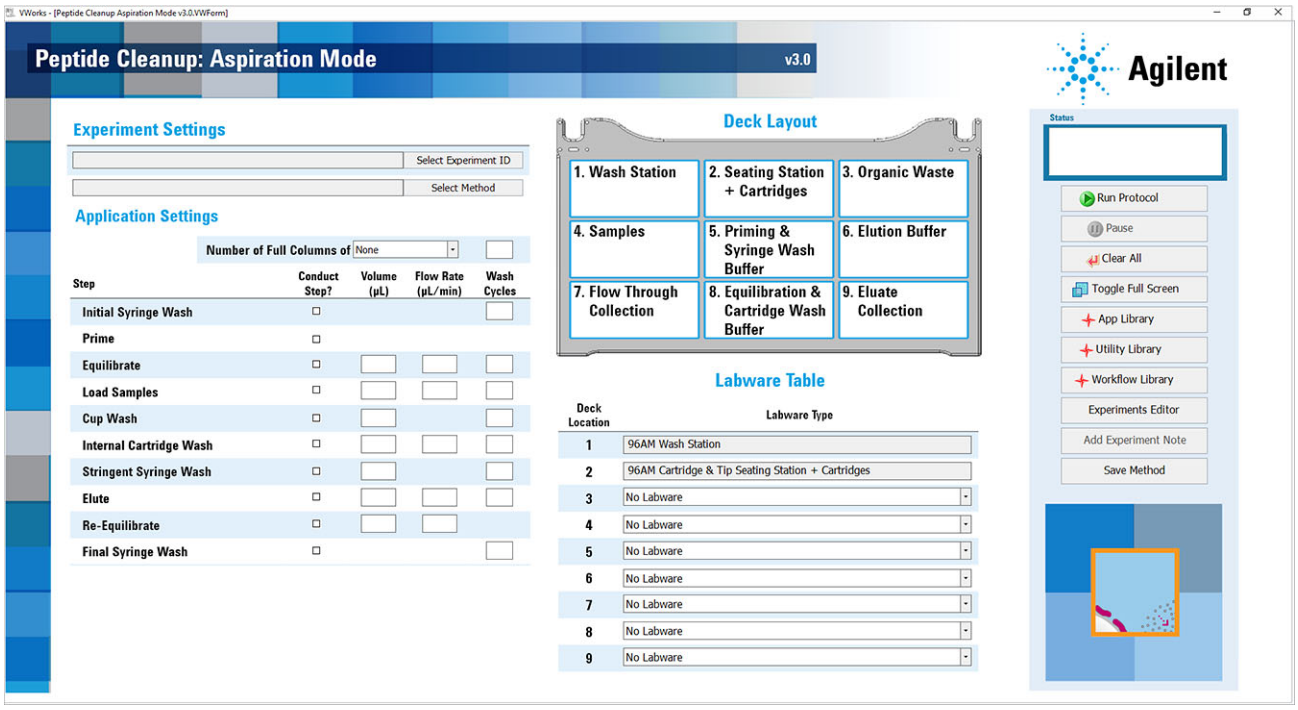
Before starting the protocol, make sure the appropriate selections and values are specified in the Peptide Cleanup: Application Mode application.

To set up the protocol:

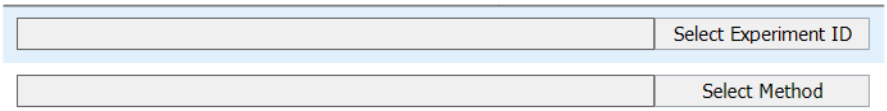
1. Open the **App Library**.
2. Locate **Peptide Cleanup: Aspiration Mode**, and then click **App**.



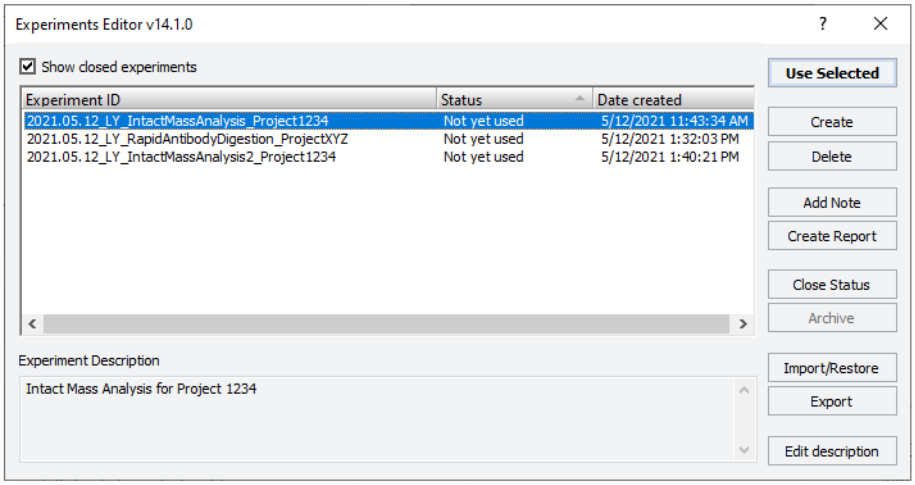
The Peptide Cleanup: Aspiration Mode application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 410.
- To modify the method, proceed to step 6.

VWorks Plus. Only VWorks administrators or technicians may modify and save methods.

6 In the **Application Settings** area, specify the **Number of Full Columns of Cartridges**.

Note: The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION

If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.

CAUTION

If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.

IMPORTANT

Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

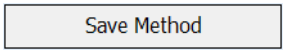
7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click 

.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 413.

Table Application Settings overview

Steps*	Description		Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 5) into the syringes, and then dispenses it through the cartridges into the Organic Waste (deck location 3).	5 µL:	100	300	–
		Range:	100	300	–
Equilibrate	Aspirates Equilibration Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the wash station (deck location 1).	5 µL:	50	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates up to 245 µL of samples (deck location 4) through the mounted cartridges into the syringes, performs an external cartridge tip wash at the wash station (deck location 1), and then aspirates a 5-µL chase of Equilibration Buffer (deck location 8). The cartridges are removed (deck location 2) and then the flow-through is dispensed into Flow Through Collection (deck location 7). Samples >245 µL are loaded in multiple steps.	5 µL:	100	5	3
		Range:	0–245	0.1–500	0–10
Cup Wash	Rinses the cartridge cups with Cartridge Wash Buffer (deck location 8), and then discards the liquid into the wash station (deck location 1).	5 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash	Aspirates Cartridge Wash Buffer (deck location 8) through the cartridges, removes the cartridges from the probes, dispenses the contents of the syringes into the wash station (deck location 1).	5 µL:	50	10	3
		Range:	0–250	0.5–500	0–10
Stringent Syringe Wash	Aspirates Syringe Wash Buffer (deck location 5) into the syringes, and then dispenses the buffer into Organic Waste (deck location 3).	5 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates Elution Buffer (deck location 6) into the syringes, and then dispenses the buffer through the cartridges into the Eluate Collection plate (deck location 9).	5 µL:	25	5	1
		Range:	0–250	0.1–500	0–10

Steps*	Description		Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Re-Equilibrate	Aspirates Equilibration Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into waste at the wash station (deck location 1).	5 µL:	50	10	–
		Range:	0–250	0.5–500	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 413.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 421.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT

The protocol will display an error messages if cartridges are missing.

Starting the protocol run

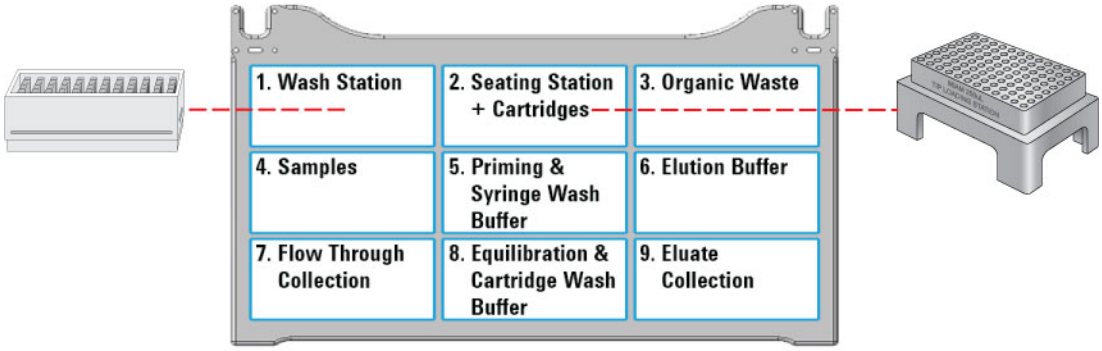
WARNING

The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

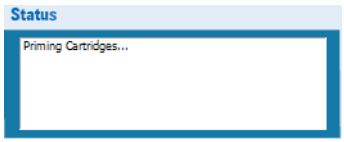
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

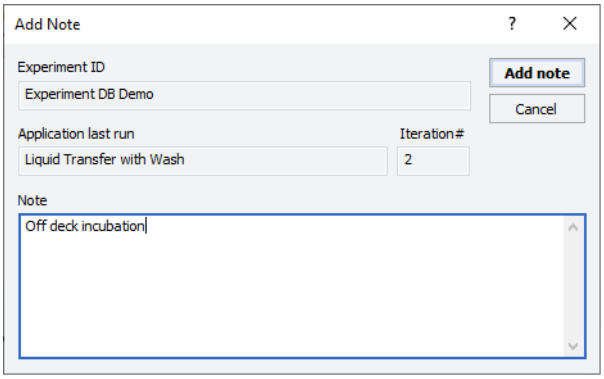
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

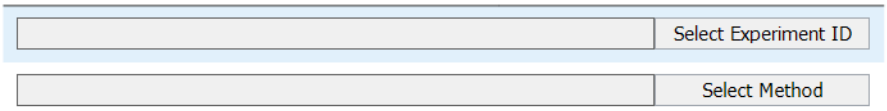
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click  to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Peptide Cleanup: Aspiration Mode protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used,* the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used,* the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12

Protocol step	Guidelines and notes
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10
Prime	This step removes entrained air from within the cartridges and properly wets the surface of the resin. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP reversed-phase cartridges (C18, RPS, and RPW) used with the Peptide Cleanup application require a Priming Buffer containing at least 25% organic solvent. As illustrated in Agilent app note 5991-6478EN ("Reference library" on page 423), reversed-phase cartridges (C18, RPS, and RPW) that are primed with solutions containing a low percentage of organic solvent have greatly reduced binding capacity. Agilent recommends using a priming solution that contains at least 50% of an organic solvent such as acetonitrile. This step is selected by default. **Volume (µL)**: • Default: 100 • Range: 100 **Flow Rate (µL/min)**: • Default: 300 • Range: 300

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the wash station. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP reversed-phase cartridges (C18, RPS, and RPW) typically used with the Peptide Cleanup application require an equilibration solution that has a very low concentration of or no organic solvent for effective binding during the sample loading step. This step is selected by default. **Volume (µL)**. The default volume is equal to 10-column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow Rate (µL/min)**. A flow rate slower than the default will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min using the default volume may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash Cycles:** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. This step mounts the cartridges on the syringes, aspirates the samples through the cartridges, and performs an external cartridge tip wash at the wash station to remove any sample on the outside of the cartridge tips. Then 5 µL of Equilibration Buffer (sample chase) is aspirated through the cartridges at the Load Samples flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. The cartridges are parked at the seating station and the flow-through plus sample chase are collected in the Flow Through Collection plate. The protocol accommodates sample volumes up to 245 µL. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. **IMPORTANT** Be sure to include the recommended labware-dependent volume overage to prevent air from entering the cartridge. For more information, see "Transferring the samples to the microplate" on page 404. This step is selected by default. **Volume (µL)**. The volume of sample should be balanced with the sample concentration and the mass capacity of the cartridge. To determine the volume of sample to load, see "Determining the volume of sample to load" on page 403. • Default: 100 • Practical: 25–245 • Range: 0–245 *Note:* The lower the sample volume the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min).** The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than the default may not significantly increase analyte binding, and using flow rates faster than the default will decrease the quantitative binding capacity of the cartridges. • Default: 5 • Practical: 2–15 • Range: 0.1–500 **Wash Cycles.** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Cup Wash	This step removes the residual sample liquid that may remain above the resin bed after the Load Samples step. The Cup Wash step aspirates Cartridge Wash Buffer into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the wash station at an offset from the chimneys, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL).** Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash Cycles:** Each wash cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash	This step uses Cartridge Wash Buffer to wash non-specifically bound molecules from the resin bed. For the wash operation, this step mounts the cartridges, aspirates Cartridge Wash Buffer through the cartridges, washes the exterior of the cartridges, parks the cartridges, and then dispenses the contents of the syringes into the wash station at an offset from the chimneys. The syringes are then washed at the wash station. **Volume (µL).** Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow Rate (µL/min).** A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash Cycles.** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Stringent Syringe Wash	This step cleans the syringes with the Syringe Wash Buffer prior to elution. The Stringent Syringe Wash step aspirates the Syringe Wash Buffer and then dispenses the buffer into the Organic Waste plate. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL):** Volumes higher than the default volume may improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash Cycles.** A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Elute	This step uses Elution Buffer to elute bound peptides from the cartridges. The Elute step aspirates the Elution Buffer into the syringes, washes the probes at the wash station, and then mounts the cartridges and dispenses the buffer through the cartridges at the specified flow rate into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges, and then the cartridges are parked at the seating station. After the elution, the samples are mixed in the Eluate Collection plate and the syringes are washed at the wash station. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples will not be mixed. This step is selected by default. **Volume (µL).** The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. The default volume is conservative and significantly higher than the minimum expected with a strong Elution Buffer. • Default: 25 • Practical: 10–30 • Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing. **Flow Rate (µL/min).** A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more buffer may be required to get the same elution yield at high flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500 **Wash Cycles.** The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Re-Equilibrate	This step uses Equilibration Buffer to return the cartridge to a low percentage organic solvent condition. The step aspirates the buffer into the syringes, mounts the cartridges, dispenses the buffer through the cartridges into the wash station, and then parks the cartridges. **Volume (µL)**. The default volume is equal to 10-column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Re-Equilibrate tasks except syringe washing. **Flow Rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min using the default volume may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. During each Final Syringe Wash cycle, the head aspirates 250 µL of DI water into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. *Note:* In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Peptide Cleanup: Aspiration Mode protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Start protocol	2	Parks all cartridges that might be on the head from a previously aborted protocol.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes.
Prime	5	Aspirates the Priming Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Priming Buffer through the cartridges and into the Organic Waste plate to prime the cartridges.
	2	Parks the cartridges.
	1	Washes the exterior of the syringe probes.
Equilibrate	8	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	1	Dispenses the Equilibration Buffer through the cartridges to equilibrate.
	1	Washes the exterior of the cartridges.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Load Samples	2	Mounts the cartridges on the head.
	4	Aspirates the samples through the cartridges into the syringes.
	1	Washes the exterior of the cartridge tips.
	8	Aspirates 5 µL of Equilibration Buffer for the sample chase.
	2	Parks the cartridges in the seating station.
	7	Dispenses the flow-through plus sample chase into the Flow Through Collection plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Cup Wash	8	Aspirates the Cartridge Wash Buffer.
	2	Washes the cartridge cups and exercises the cartridges off task.
	1	Dispenses the Cartridge Wash Buffer into the wash station.
	1	Washes the syringes.
Internal Cartridge Wash	2	Mounts the cartridges on the head.
	8	Aspirates the Cartridge Wash Buffer through the cartridges into the syringes.
	1	Washes the exterior of the cartridges.
	2	Parks the cartridges.
	1	Dispenses the Cartridge Wash Buffer into the wash station, and then washes the exterior of the syringe probes.
Stringent Syringe Wash	5	Aspirates the Syringe Wash Buffer.
	3	Dispenses the syringe contents into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
Elute	6	Aspirates the Elution Buffer into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	9	Elutes the samples into the Eluate Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
	9	Mixes the eluates.
	1	Washes the syringes.
Re-Equilibrate	8	Aspirates Equilibration Buffer into the syringes.
	2	Mounts the cartridges on the head.
	1	Dispenses the Equilibration Buffer through the cartridges to equilibrate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
Final Syringe Wash	1	Washes the syringes.

Reference library

1 Russel, J., Van Den Heuvel, Z., Bovee, M. & Murphy, S., Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-2957EN, 2016

2 Bovee, M., Krahenbuhl, A. & Murphy, S., Rapid Antibody Digestion Enabled by Automated Reversed-Phase Desalting on the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-6478EN, 2016

See the Agilent AssayMAP Bravo Citation Index for published papers that used the AssayMAP Bravo Platform.

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13 Phosphopeptide Enrichment v3.0 User Guide

This chapter contains the following topics:

App description

Phosphopeptide Enrichment v3.0. This application enables automated phosphopeptide enrichment on from 1 to 96 samples in a single run.

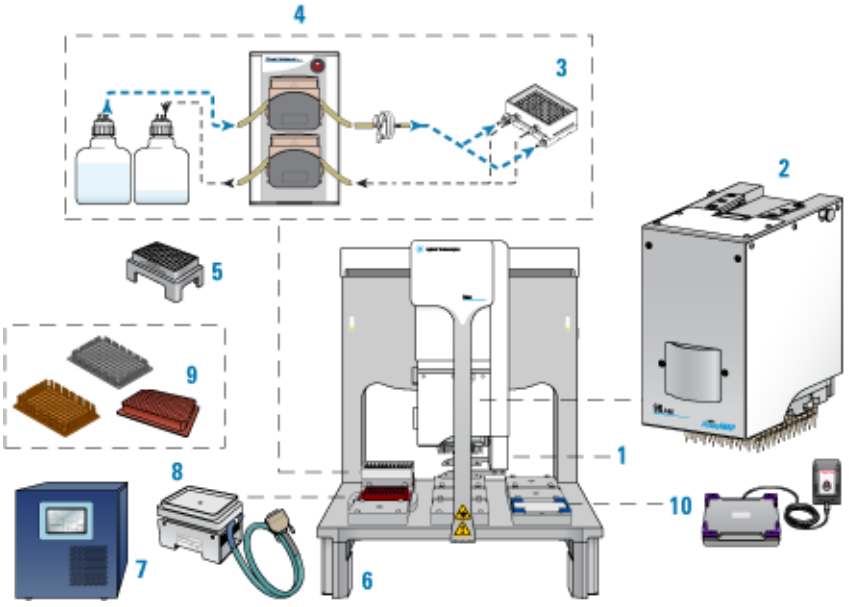
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Phosphopeptide Enrichment protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which is required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. You might need the following when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate vacuum concentrator for concentration and drying of samples
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Phosphopeptide Enrichment on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Fe(III)-NTA cartridge rack	G5496-60085	–
AssayMAP TiO_2 cartridge rack	G5496-60016	–
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or to fill remaining positions in columns that are partially occupied by Fe(III)-NTA or TiO_2 cartridges, if necessary. For details, see "Preparing the samples" on page 434.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT

Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.
- AssayMAP TiO_2 cartridges are stable from approximately pH 1 to 14.

- AssayMAP Fe(III)-NTA cartridges are stable from approximately pH 2 to 11. At levels approximately > pH 3.5, resin within the cartridges may turn from pale yellow to golden yellow to orange or brown due to the formation of iron(III) complexes (most commonly with hydroxide). Resin coloration and intensity are a function of both the pH and the chemical nature of the solutions passed through the cartridge. Bare NTA cartridges are stable from approximately pH 2 to 14.
- AssayMAP Fe(III)-NTA cartridges that have been stripped (bare NTA cartridges) should be charged and used immediately.

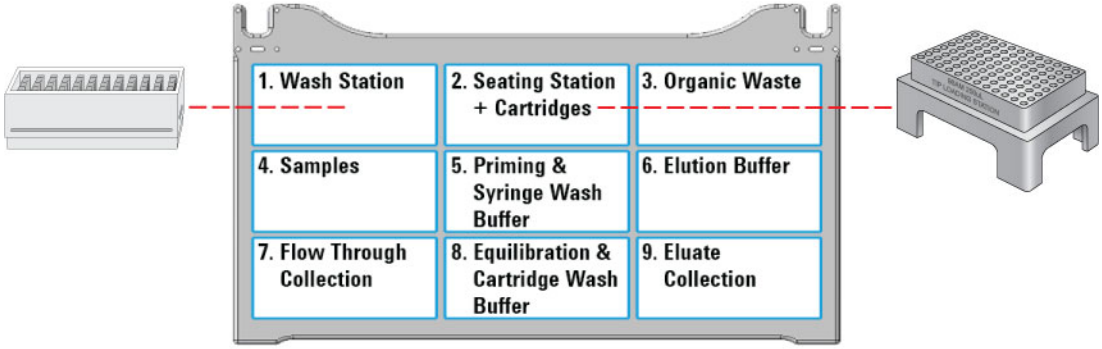
Labware

Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3, 5–8
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3, 5–8
96 Agilent, 2mL Square Deep Well labware	Agilent 204353-100	3, 7
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3–8
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7, 9**
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	4, 7, 9**
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3–9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3–9

Labware	Manufacturer part number*	Deck location options
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3–9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3–9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	4, 7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	5, 6, 8
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	5, 6, 8
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottoms	Agilent 201254-100	5, 6, 8

*For dimensionally equivalent alternatives and other labware details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner and BioRad PCR plates are not compatible with the 25 µL cartridges at deck locations 7 and 9.

Reagents

The volume, type, and concentration of reagents and buffers required for phosphopeptide enrichment will vary depending on the specific AssayMAP phosphopeptide enrichment cartridge being used, the sample characteristics, and the desired analytical result. Consult Agilent app note 5991-6073EN ("Reference library" on page 462) for reagent recommendations for using the AssayMAP Fe(III)-NTA with the Phosphopeptide Enrichment application. The reference library also contains references to published papers for using both Fe(III)-NTA and TiO2 for phosphopeptide enrichment. See the Agilent AssayMAP Bravo Citation Index for published papers that use the Phosphopeptide Enrichment application on the AssayMAP Bravo Platform to purify phosphopeptides from a wide range of sample matrices.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 430.

Preparing the solutions

The following solutions are required for the Phosphopeptide Enrichment protocol:

- Priming Buffer and Syringe Wash Buffer

- Equilibration and Cartridge Wash Buffer
- Elution Buffer

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Phosphopeptide Enrichment

The Reagent Volume Calculator is a Microsoft Excel file that contains a Calculator worksheet. You enter the number of columns to process, whether to perform the Collect Flow Through options, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.

To use the Reagent Volume Calculator:

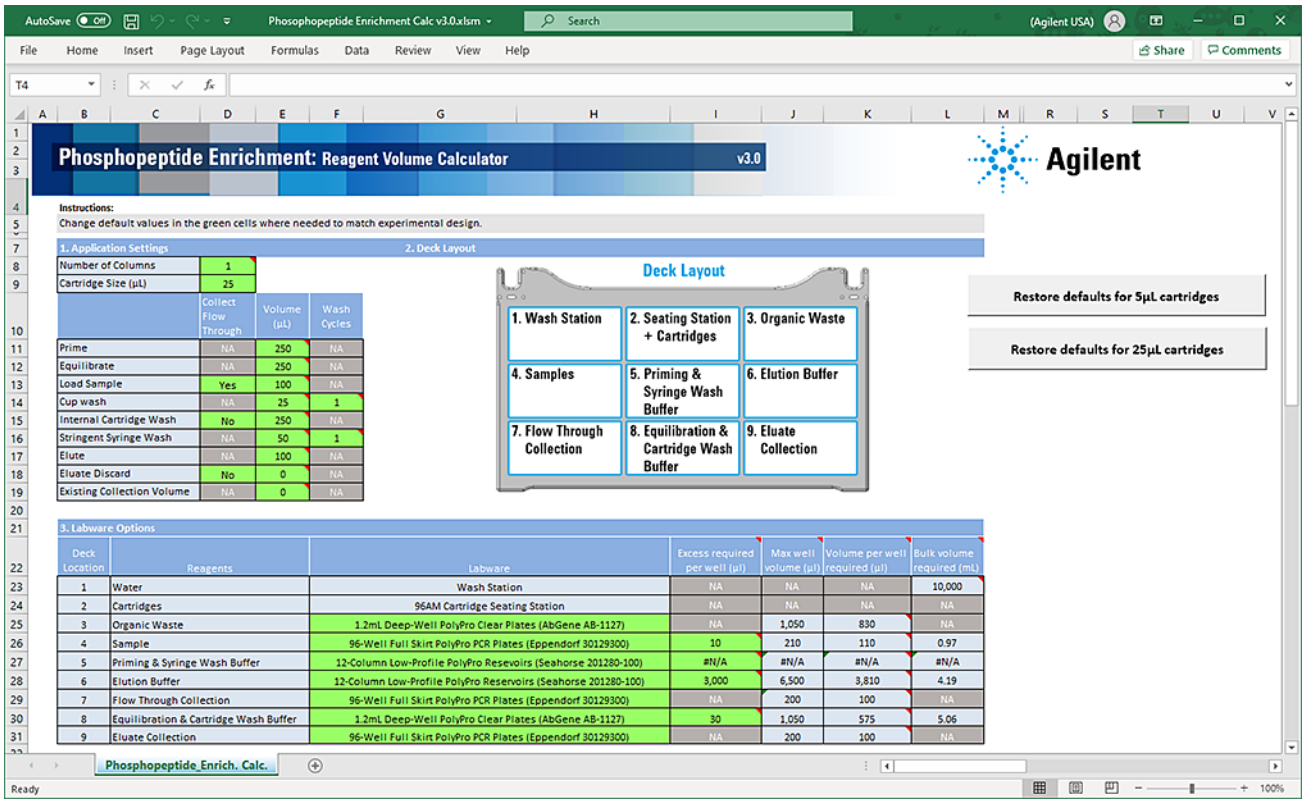
1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figure shows the Reagent Volume Calculator.

Figure Phosphopeptide Enrichment Calculator worksheet



Preparing the buffers

CAUTION **Make sure you filter salt-containing buffers if salt precipitation is a risk. Salt precipitates can clog cartridges and cause poor performance.**

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the following buffers as a **starting point** for optimizing the AssayMAP phosphopeptide enrichment chemistry.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming Buffer & Syringe Wash Buffer (deck location 5)	This dual-purpose buffer serves as both the Priming Buffer and Syringe Wash Buffer during the protocol run. Do not alter the composition without evaluating how the change affects the priming step. Organic solvent in the buffer helps to purge entrained air within the cartridge resin bed during priming and is effective at removing hydrophobic species from syringes as part of the stringent syringe wash. Priming with a high-pH buffer helps to prepare TiO_2 cartridges better than priming with a low-pH buffer. The following buffers have been used successfully with the indicated cartridges. **Fe(III)-NTA**: 99.9% ACN : 0.1% TFA **TiO2** : 50% ACN : 45% H2O : 5% NH3
Elution Buffer (deck location 6)	Dilute solutions of aqueous ammonia are sufficient to elute phosphopeptides from Fe(III)-NTA and TiO_2 cartridges. Recovery of hydrophobic peptides may be improved with the addition of small percentages of acetonitrile. The following buffers have been successfully used with the indicated cartridges. **Fe(III)-NTA**: 99 % H2O : 1% NH3 **TiO2**: 80 % H2O : 15% ACN : 5% NH3
Equilibration & Cartridge Wash Buffer (deck location 8)	This solution should be similar in composition to the sample loading buffer. The following buffers have been successfully used with the indicated cartridges. **Fe(III)-NTA**: 80% ACN : 19.9% H2O : 0.1% TFA **TiO2**: 50% ACN : 48 % H2O : 2% TFA

Note: All suggested solutions listed as percentages are volume/volume formulations.

Note: The source of NH_3 for these buffers is aqueous ammonium hydroxide, which is commonly listed as $\%NH_3$ in H_2O from commercial suppliers.

Dispensing the solutions

IMPORTANT

To prevent evaporation, dispense the reagents into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT

If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and the cartridge positions in the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 434.

To dispense the solutions into the labware:

1. *Optional*. Label the labware so that you can easily identify them.
2. Add the specified volume of Priming & Syringe Wash Buffer into the labware to be placed at deck location 5.
3. Add the specified volume of Elution Buffer into the labware to be placed at deck location 6.
4. Add the specified volume of Equilibration & Cartridge Wash Buffer into the labware to be placed at deck location 8.
5. If necessary, centrifuge the reagent labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT

To minimize evaporation, prepare the samples immediately before running the Phosphopeptide Enrichment protocol, or keep the plates lidded until the run begins.

When preparing the samples, you must:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to optimize the binding conditions.
- Determine the volume of samples to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulates

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the sample composition

Optimal conditions for phosphopeptide binding to AssayMAP phosphopeptide enrichment cartridges

In a review of relevant literature for phosphopeptide enrichment using various titanium dioxide formats (2–28) and immobilized metal affinity chromatography (IMAC) formats (8, 29–55), there was no clear consensus on sample and reagent formulations that yielded the optimum phosphopeptide enrichment ratio, the highest absolute phosphopeptide recovery, and the greatest reproducibility. In the majority of these studies, reagent formulations were optimized for the experiment at hand.

Similarly, phosphopeptide enrichment using AssayMAP Fe(III)-NTA and TiO_2 cartridges requires some degree of chemistry optimization and method development depending on the desired analytical result. No one set of chemistry conditions will be universally optimal. However, there are some chemistry trends that can serve as reasonable starting points for method development.

Typically, samples for phosphopeptide enrichment using Fe(III)-NTA and TiO_2 have first been subjected to a reversed-phase cleanup (desalting) step. Samples are generally in a phosphate-free, TFA-containing, low-pH buffer (pH 2.4 to 2.8) with some amount of organic solvent. Low-pH conditions reduce non-specific binding of non-phosphopeptides by facilitating protonation of acidic side-chains and C-termini of peptides, whereas the phosphoryl groups of phosphopeptides remain negatively charged at low pH to promote binding in a combination of ion exchange and metal coordination to the stationary phase (2, 56). The presence of organic solvent (typically 25% to 80% acetonitrile) in the sample and wash solutions helps to reduce nonspecific binding of non-phosphopeptides. Organic solvent serves to modulate the pKa values of ionizable species and the effect can be very different depending on the chemical species (57, 58). Achieving high phosphopeptide enrichment often means finding conditions that maximize the difference in the pKa values of phosphoryl and carboxyl groups such that under a given set of solvent conditions, the phosphoryl groups are negatively charged while carboxyl groups are neutral (45, 49). This is accomplished by adjusting the type and concentration of both the acid modifier and organic solvent present in the samples and wash buffers. Specificity can also be improved for phosphopeptide enrichment by TiO_2 with the addition of small-molecule organic acids (such as, glycolic acid, lactic acid, and dihydroxybenzoic acid), which may further reduce nonspecific binding of non-phosphopeptides by competitively displacing weakly bound acidic, non-phosphopeptides.

Sample components that cause concerns

Samples should be free of phosphates, free of particulates, and contain no or low concentrations of salts, denaturants, and chaotropes. The best results are typically achieved using samples first subjected to reversed-phase cleanup (desalting) before phosphopeptide enrichment. Solubilization of peptide samples after desalting and drying can be problematic when using high levels of organic solvent in the sample solubilization or reconstitution buffer. Solubility is often promoted by first adding the aqueous component of the buffer to the sample along with thorough mixing. This is followed by a slow titration of the organic portion of the buffer into the sample until the desired aqueous or organic ratio is achieved.

Poor enrichment efficiency can be caused by insoluble or precipitated peptides that can form particulates large enough to collect at the top of the resin bed in the cartridge during sample loading. These particulates will mostly likely contain a very high percentage of non-phosphopeptides. If the wash buffer is insufficient to solubilize the particulates collected at the top of the cartridge, it is likely that the high-pH, aqueous

conditions used for elution will solubilize the particulates. The peptides from the particulates will co-elute with the phosphopeptides and decrease the percent enrichment. If sample particulates form post filtration, attempt to refilter the sample or pellet the particulates by centrifugation before sample loading.

Sample load volume and cartridge capacity

The AssayMAP Phosphopeptide Enrichment protocol permits loading of up to 1000-µL sample volumes onto AssayMAP phosphopeptide enrichment cartridges. For sample volumes > 250 µL, the protocol will iteratively load samples onto cartridges to stay within the maximum AssayMAP syringe volume (250 µL).

For each AssayMAP Fe(III)-NTA and TiO_2 cartridge, the quantitative binding capacity (≥ 90% recovery) depends on the chemical characteristics of the phosphopeptide targets and the sample matrix. Matrix effects include the overall sample complexity and the presence and abundance of certain of solvents, buffers components, additives, and the pH of the sample.

For a tryptic digest of bovine a-casein, ~10 mole phosphate per mole protein (59), quantitative recovery of phosphopeptides is achieved with up to 1200-µg digest (~500 nmol phosphate) loaded onto TiO_2 cartridges and up to 150-µg digest (~ 65 nmol phosphate) loaded onto Fe(III)-NTA cartridges.

AssayMAP Fe(III)-NTA cartridges have an iron content greater than 100 nmol Fe(III) per cartridge as determined by inductively coupled plasma—optical emission spectroscopy (ICP-OES).

For enrichment of the small molecule test substrate phenylphosphate (4, 27), the cartridge capacity at saturation is approximately:

- 115 µg (~ 660 nmol) per TiO_2 cartridge
- 16 µg (~ 92 nmol) per Fe(III)-NTA cartridge

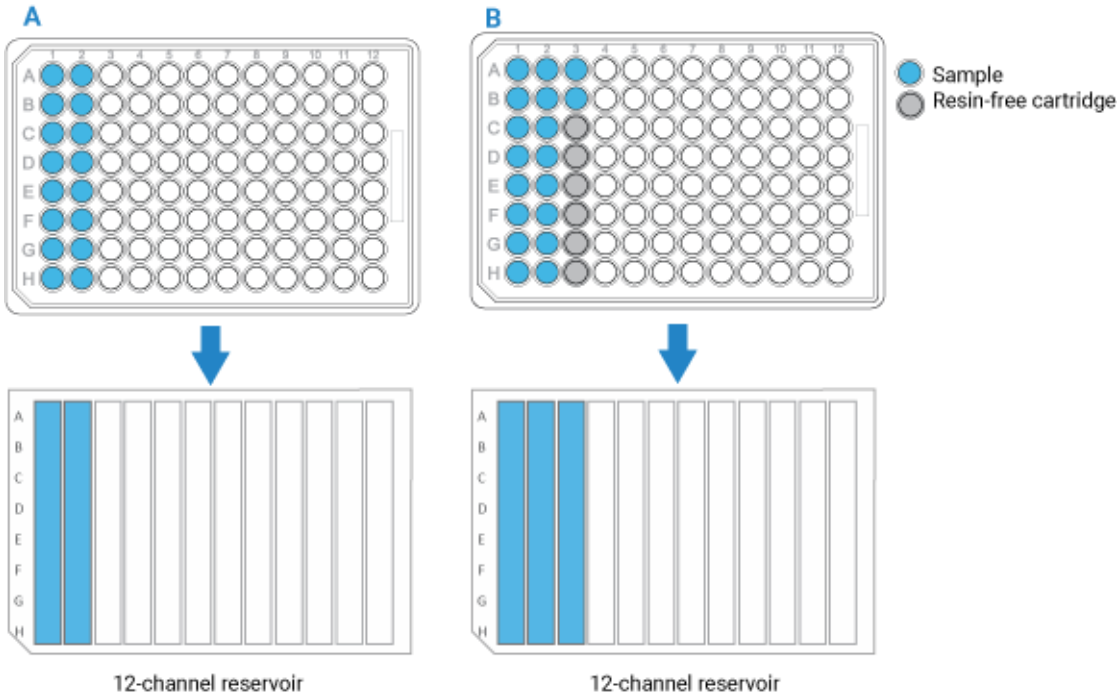
Preparing the sample plates

Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must also match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the figure below shows.
- The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based upon the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use the AssayMAP Resin-Free cartridges to fill the empty column and row positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash step.

Figure Example of sample microplate and reservoir layout: A) Multiple of 8 samples, B) Not a multiple of 8



See "Labware" on page 429 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION

A small volume excess is required in all labware types to ensure proper volume transfer.

An excess (overage) volume ensures that a microplate well does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well. See "Using the Reagent Volume Calculator for Phosphopeptide Enrichment" on page 431.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide,* which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Phosphopeptide Enrichment protocol:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the peptides from the cartridges.

For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the cartridge seating station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reagents. See "Preparing the solutions" on page 430.
- Prepare the samples. See "Preparing the samples" on page 434.

- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup protocol to prepare the AssayMAP Bravo Platform for the run. See the System Startup/Shutdown v3.0 User Guide utility.
- Transfer the cartridges to the Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the cartridge bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

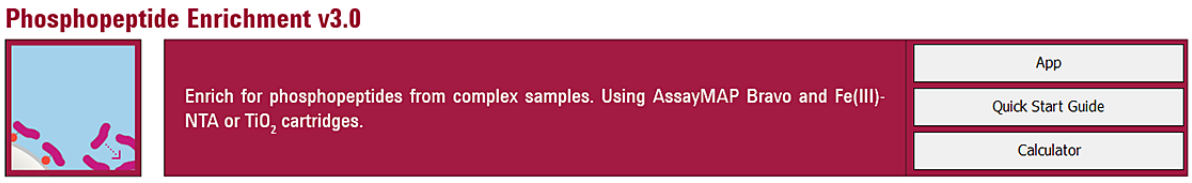
IMPORTANT Do not allow wetted cartridges to dry out. Agilent Technologies does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 428.

Setting up the protocol

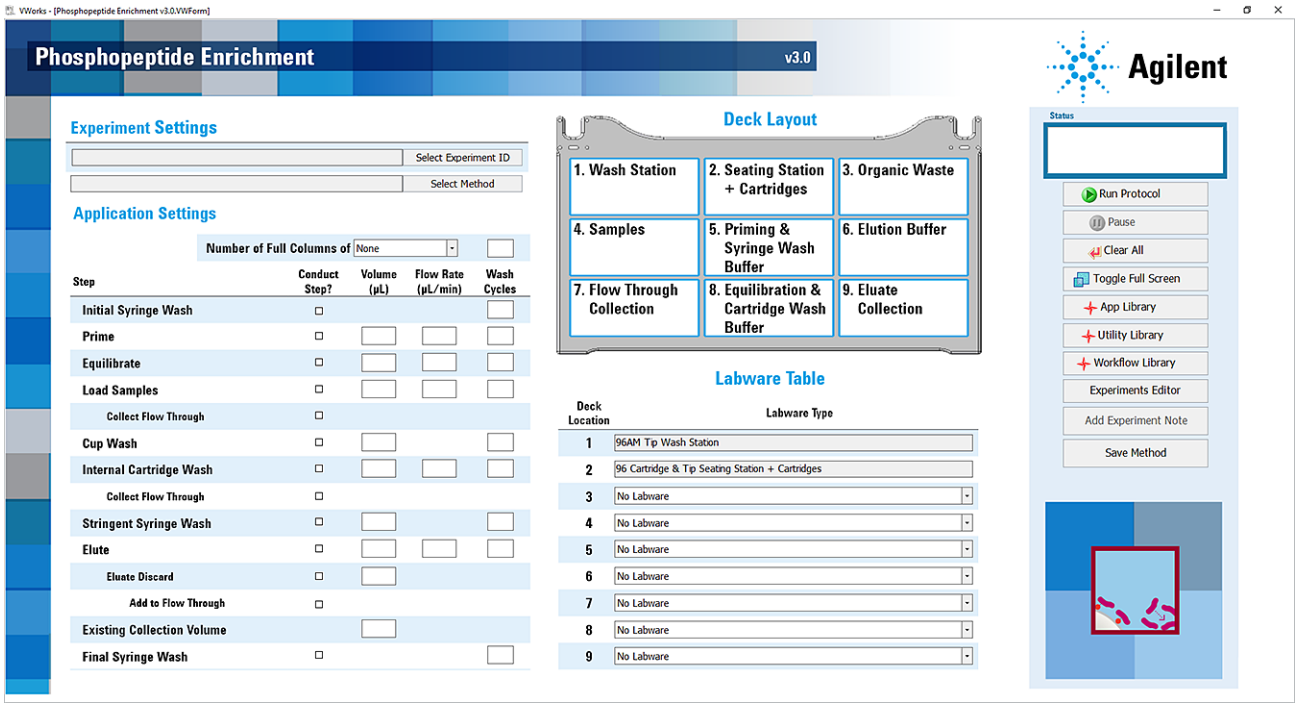
Before starting the protocol, make sure the appropriate selections and values are specified in the Phosphopeptide Enrichment application.

To set up the protocol:

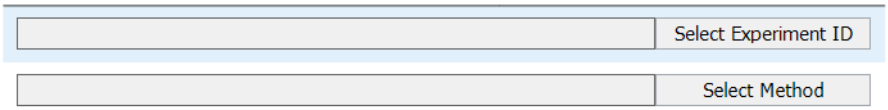
1. Open the **App** library.
2. Locate **Phosphopeptide Enrichment**, and then click **App**.



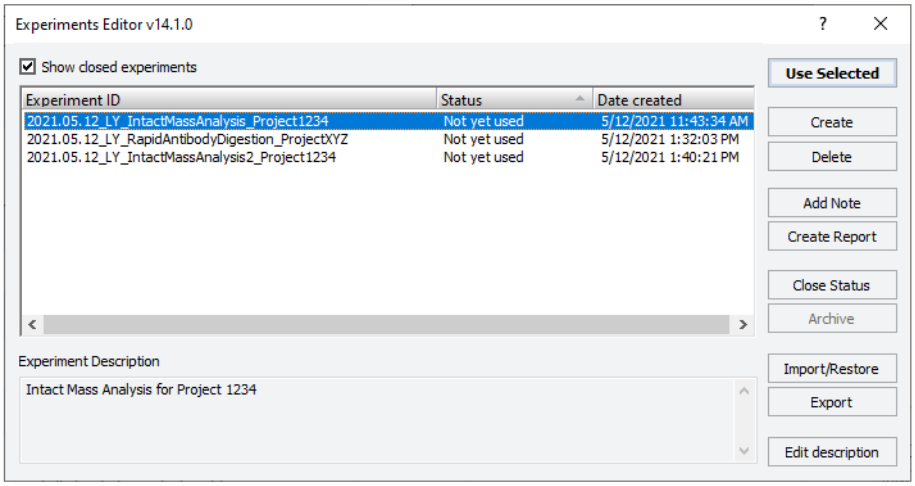
The Phosphopeptide Enrichment application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 444.
- To modify the method or create a method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:

a Select the cartridge size from the list:
- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION

If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.

CAUTION

If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.

IMPORTANT

Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 446.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 5) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10
Equilibrate	Aspirates Equilibration Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates samples (deck location 4) into the syringes, and then dispenses them through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 7).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–1000	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the sample flow-through in the Organic Waste plate (deck location 3).	–	–	–	–
Cup Wash	Rinses the cartridge cups with Cartridge Wash Buffer (deck location 8), and then discards the liquid into the Organic Waste plate (deck location 3).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Internal Cartridge Wash	Aspirates Cartridge Wash Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 7).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash flow-through at the Flow Through Collection (deck location 7). If not selected, discards the Internal Cartridge Wash flow-through into the Organic Waste (deck location 3).	–	–	–	–
Stringent Syringe Wash	Aspirates Syringe Wash Buffer (deck location 5) into the syringes, and then dispenses it into the Organic Waste plate (deck location 3).	5 µL:	50	–	2
		25 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates Elution Buffer (deck location 6) into the syringes, and then dispenses it through the cartridges into the Eluate Collection plate (deck location 9).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0–250	0.1–500	0–10
Eluate Discard	If selected, a specified initial volume of the eluate is discarded into the Organic Waste plate (deck location 3), or collected in the Flow Through Collection plate (deck location 7).	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–250	–	–
Add to Flow Through	If selected, collects the Eluate Discard in the Flow Through Collection plate (deck location 7). If not selected, discards the Eluate Discard into the Organic Waste plate (deck location 3).	–	–	–	–
Existing Collection Volume	Specifies the volume of liquid present in the Eluate Collection plate (deck location 9) at the beginning of the run.	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–300	–	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see the "Protocol stepwise guidelines" on page 447.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 458.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT The protocol will display an error message if cartridges are missing.

Starting the protocol run

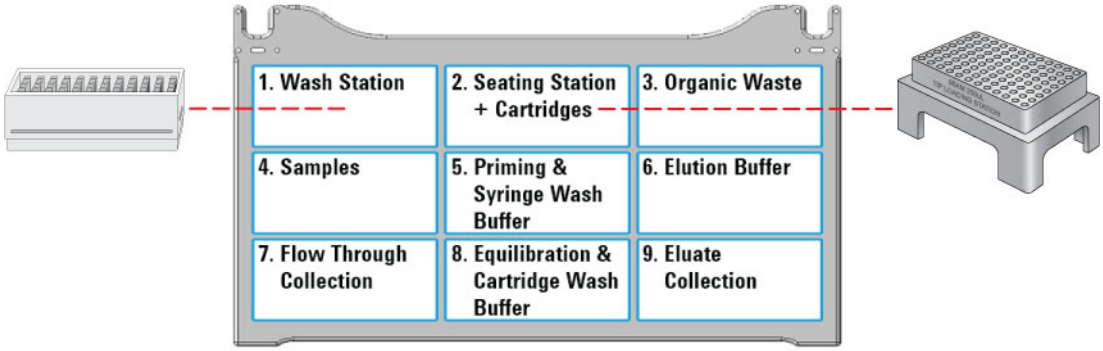
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

Note: The Greiner and BioRad PCR plates are not compatible with the 25 µL cartridges at deck locations 7 and 9.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

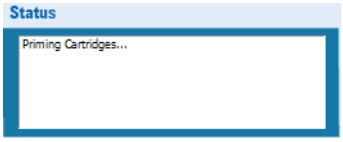
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol. The default protocol with 100-µL samples and 5-µL/min sample loading flow rate should take approximately 50 minutes to complete.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

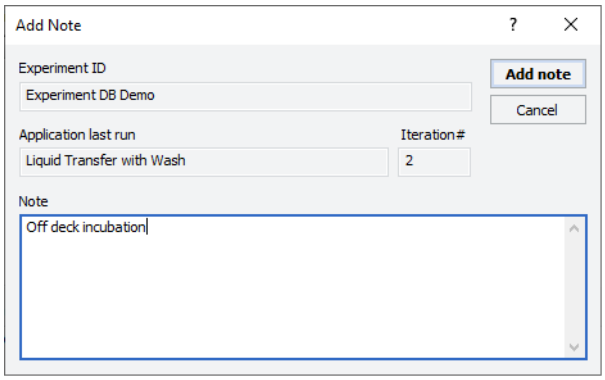
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



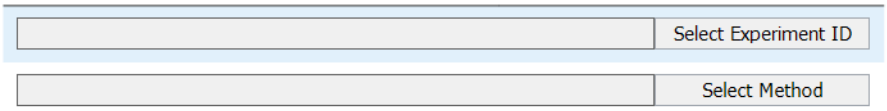
2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

1. Remove used labware from the deck.
2. Discard leftover reagents appropriately.
3. *Optional*. Conduct stringent washing of the syringes:
   a. Open the **Syringe Wash** utility  .
   b. If applicable, click **Select Experiment ID** to open the Experiments Editor.



   c. In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.
   d. Click **Select Method** to select and load the method for this utility.
   e. Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.
   f. Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Phosphopeptide Enrichment protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12
Initial Syringe Wash	This step flushes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the resin bed and properly wets the surface of the resin. In preparation for priming, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. For the most effective priming of AssayMAP Fe(III)-NTA and TiO_2 cartridges, the Priming Buffer requires that the solution contain at least 50% organic solvent. Higher concentrations of organic solvent are also acceptable. This step is selected by default. **Volume (µL)**. The default volume is sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default value diminishes the ability to effectively remove entrained air from the resin bed. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. *Note:* The Equilibration Buffer is also used for the Cartridge Wash Buffer. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the sample loading begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates sample into the syringes, and then performs an external syringe wash at the wash station to remove any sample remaining on the outside of the probes before mounting the cartridges. The samples are dispensed through the cartridges into the Flow Through Collection plate or the Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any sample on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. The protocol accommodates sample volumes up to 1000 µL to be dispensed through the AssayMAP phosphopeptide enrichment cartridges. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. Each syringe has a maximum capacity of 250 µL. When sample volumes are greater than 250 µL, the protocol will iteratively load samples through the cartridges. To determine the number and volume of iterative load steps, the protocol uses the following formulas: • *# of times to load* = total sample volume/250, where # times to load is rounded up to nearest integer • *volume of each load* = sample volume/# of times to load For example, if the total sample volume is 900 µL, then: *# times to load* = 900/250 = 3.6, which is rounded up to 4 *volume of each load* = 900/4 = 225 If Collect Flow Through is selected for the Load Samples step, be sure that the Flow Through Collection plate has sufficient maximum well capacity. For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. **IMPORTANT** Be sure to include the recommended labware-specific volume overage to prevent air from entering the cartridge. For more information, see "Preparing the sample plates" on page 436. To determine the volume of sample to load, see "Sample load volume and cartridge capacity" on page 436. This step is selected by default.

Protocol step	Guidelines and notes
	Volume (µL). The volume of sample should be balanced with the sample concentration and the mass capacity of the cartridge. Large sample volumes (> 250 µL) may require slightly more excess sample due to evaporation. • Default: 100 • Practical: 10–1000 • Range: 0–1000 *Note:* The lower the sample volume the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min)**. The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than 5 µL/min may not significantly increase analyte binding. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed into the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step is dispensed into the Organic Waste plate. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is selected by default.

Protocol step	Guidelines and notes
Cup Wash	This step removes the residual sample solution that may remain above the resin bed after the Load Samples step. The Cup Wash step aspirates Cartridge Wash Buffer into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the Organic Waste plate, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10
Internal Cartridge Wash	This step uses Cartridge Wash Buffer to wash non-specifically bound molecules from the resin bed. In preparation for the Internal Cartridge Wash, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Cartridge Wash Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates Cartridge Wash Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or the Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. If the Load Samples step is selected, the first 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) of Cartridge Wash Buffer 1 is dispensed as a sample chase at the Load Samples flow rate. Next, the Internal Cartridge Wash volume minus the chase volume is dispensed at the Internal Cartridge Wash flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. This step is selected by default.

Protocol step	Guidelines and notes
Internal Cartridge Wash (continued)	**Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increase the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the flow-through from Internal Cartridge Wash step is dispensed in the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from Internal Cartridge Wash is dispensed into the Organic Waste plate. This step is not selected by default.

Protocol step	Guidelines and notes
Stringent Syringe Wash	This step cleans the syringes with the Syringe Wash Buffer prior to elution. The Stringent Syringe Wash step aspirates the Syringe Wash Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the Organic Waste plate. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume are unlikely to improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash cycle**. A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10
Elute	This step uses Elution Buffer to elute bound analytes from the cartridges. In preparation for elution, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Elution Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges, and then the cartridges are parked at the seating station. After the elution, the eluate is mixed in the Eluate Collection plate and the syringes are washed at the wash station. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples will not be mixed. You can also select the Eluate Discard and Add to Flow Through substeps, which are described in the following rows of this table. This step is selected by default.

Protocol step	Guidelines and notes
Elute (continued)	**Volume (µL)**. The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges, or 50–75 µL for 25 µL cartridges). The default volumes are conservative and significantly higher than the minimum expected with a strong Elution Buffer. *Note:* The Eluate Collection plate must be able to accommodate the total volume, which is determined by summing the net elution volume (Elute volume - Eluate Discard volume) and the Existing Collection Volume. For labware-specific maximum well volumes, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. • Volume for 5 µL cartridges: – Default: 25 – Practical: 10–30 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 125 – Practical: 50–150 – Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing. **Flow rate (µL/min).** A flow rate slower than 5 µL/min is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more elution buffer may be required to get the same elution yield at high elution flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Eluate Discard	This substep of the Elute step permits a specified volume of the eluate from the cartridges to be discarded before the eluate starts to be collected during the Elute step. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the Elution Buffer at the Elute flow rate through the cartridges. If the Eluate Discard step is selected, the specified volume is dispensed into the Organic Waste plate or Flow Through Collection plate (if the Add to Flow Through step is selected). The remaining Elution Buffer is dispensed through cartridges into the Eluate Collection plate. **Example**: If the Elute, Eluate Discard, and Add to Flow Through steps are all selected with the following settings: Elute volume = 15 µL (5 µL cartridges) or 40 µL (25 µL cartridges) Eluate Discard volume = 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) the first 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) eluate from the cartridges will be discarded into the Flow Through Collection plate, and the remaining 13 µL (5 µL cartridges) or 30 µL (25 µL cartridges) eluate will be collected in the Eluate Collection plate. Select the Eluate Discard step in situations where minimizing the volume of eluate is important. For AssayMAP cartridges, the initial elution volume (~2 µL for 5 µL cartridges and ~10 µL for the 25 µL cartridges) contains small or no measurable amounts of analyte. This step is not selected by default. **Volume (µL)**. The first volume of eluate that will be discarded during the Elute step. This value can equal, but cannot exceed the Elute volume. • Default: 0 • Practical: – 5 µL cartridges: 0–2 – 25 µL cartridges: 0–10 • Range: 0–250
Add to Flow Through	If selected, this step dispenses the Eluate Discard volume into the Flow Through Collection plate. If the Add to Flow Through step is not selected, the Eluate Discard is dispensed into the Organic Waste plate. This step is not selected by default. The Add to Flow Through step is an option only if both the Elute and Eluate Discard steps have been selected. *Note:* The Add to Flow Through step is an option only if both the Elute and Eluate Discard steps have been selected.

Protocol step	Guidelines and notes
Existing Collection Volume	This step enables you to specify an amount of liquid that is in the wells of the Eluate Collection plate at the beginning of the run. The Existing Collection Volume and the net volume from the Elute step (Elute volume - Eluate Discard volume) feeds into logic that adjusts the well-bottom offset for sample elution, calculates the eluate mixing volume, and dynamically moves the head into and out of the wells during elution and eluate mixing in a volume-dependent manner. For the maximum practical working volumes of labware for eluate collection, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. Select this step when the Eluate Collection plate contains a volume of liquid useful for immediately diluting the eluates, for adjusting the pH of the eluates, or to aid in the recovery of small volumes of eluates from AssayMAP cartridges. **Volume (µL):** • Default: 0 • Practical: 0—250 • Range: 0—300
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate. No solution is added into the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL of DI water into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. *Note:* In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Phosphopeptide Enrichment protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	2	Parks all cartridges that might have been loaded on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes.
Prime	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	5	Aspirates 10 µL of Priming Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	5	Aspirates the Priming Buffer.
	2	Mounts the cartridges onto the head.
	3	Dispenses the Priming Buffer through the cartridges into the Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	8	Aspirates 10 µL of Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Equilibration Buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Equilibration Buffer through the cartridges to equilibrate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Load Samples	4	Aspirates the samples into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	7	Dispenses the samples through the cartridges and into the Flow Through Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash	8	Aspirates the Cartridge Wash Buffer into the syringes.
	2	Washes the cartridge cups and exercises the cartridges off task.
	3	Dispenses the Cartridge Wash buffer into the Organic Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	8	Aspirates 10 µL of Cartridge Wash Buffer 1 for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Cartridge Wash Buffer into the syringes for sample chase and the Internal Cartridge Wash steps.
	2	Mounts the cartridges on the head.
	3	Dispenses 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) Cartridge Wash Buffer through the cartridges at the Load Samples flow rate for the sample chase step.
	3	Dispenses the remaining Cartridge Wash buffer through the cartridges at the Internal Cartridge Wash flow rate and into the Organic Waste plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Stringent Syringe Wash	5	Aspirates the Syringe Wash Buffer into the syringes.
	3	Dispenses the Syringe Wash Buffer into the Organic Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Elute	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	6	Aspirates 10 µL of Elution Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Elution Buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates the Elution Buffer.
	2	Mounts the cartridges on the head.
	9	Elutes the samples into the Elution Collection Plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	9	Mixes the eluates.
	1	Washes the syringes.
Final Syringe Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the syringes.

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See the Agilent AssayMAP Bravo Citation Index for published papers that used the AssayMAP Bravo Platform.

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14 Protein Cleanup v3.0 User Guide

This chapter contains the following topics:

App description

Protein Cleanup v3.0. This application enables automated cleanup of from 1 to 96 protein samples in a single run.

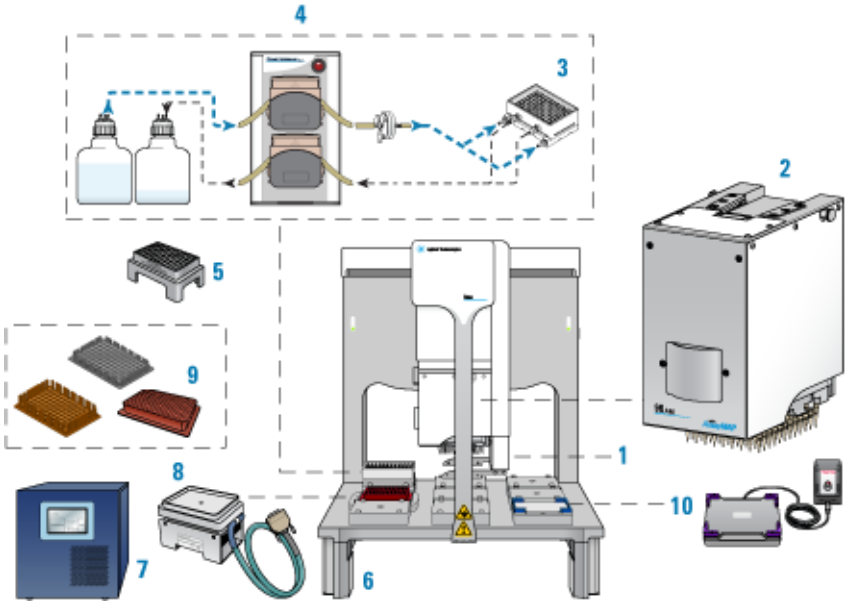
Before you start

This topic lists the required hardware, software, AssayMAP cartridges, labware, and reagents for running the Protein Cleanup protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Optional equipment. You might need the following when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate vacuum concentrator for concentration and drying of samples
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP cartridges

The following table lists the available AssayMAP cartridges for performing Protein Cleanup on the AssayMAP Bravo Platform. Each cartridge type can be purchased as a rack of 96 cartridges.

Cartridge type	Agilent part number	
	5 µL cartridge	25 µL cartridge
AssayMAP Reversed-Phase (RP-W) cartridge rack	G5496-60086	G5496-60024
AssayMAP Resin-Free cartridge rack This cartridge can be used for mock runs or as cartridge placeholders if only partial columns of RP-W 5- or 25-µL cartridges are required. For more information, see "Preparing the samples" on page 476.	G5496-60009	–

For more details about the available cartridges, see the Agilent AssayMAP Bravo Cartridges Selection Guide or the AssayMAP Cartridges page on Agilent.com.

Cartridge use and storage guidelines

See the cartridge box label for storage guidelines.

Follow these guidelines to get the best performance from AssayMAP cartridges:

- Use only primed and equilibrated cartridges.

IMPORTANT Cartridges ship dry and, therefore, contain air entrained in the resin bed. Failure to prime the cartridges can prevent the reagents and buffers from accessing parts of the resin bed, resulting in reduced capacity and poor reproducibility.

- Do not allow wetted cartridges to dry out.

  Note: Cartridges will not dry out during the course of a normal application run. Cartridges can dry out if they are exposed to air for extended periods (e.g., >1 hour) after they have been primed and equilibrated.

  If you need to store primed and equilibrated cartridges for a short period, ensure that you use the lidded blue rack-receiver plate stack with an appropriate solution in the receiver plate chimneys such that the cartridge tips are submerged in the solution.
- AssayMAP cartridges are intended to be single-use consumables. Agilent does not provide a performance guarantee for cartridges that have been used more than once.
- AssayMAP RP-W cartridges tolerate brief exposure to solutions with pH values of approximately 1–14.

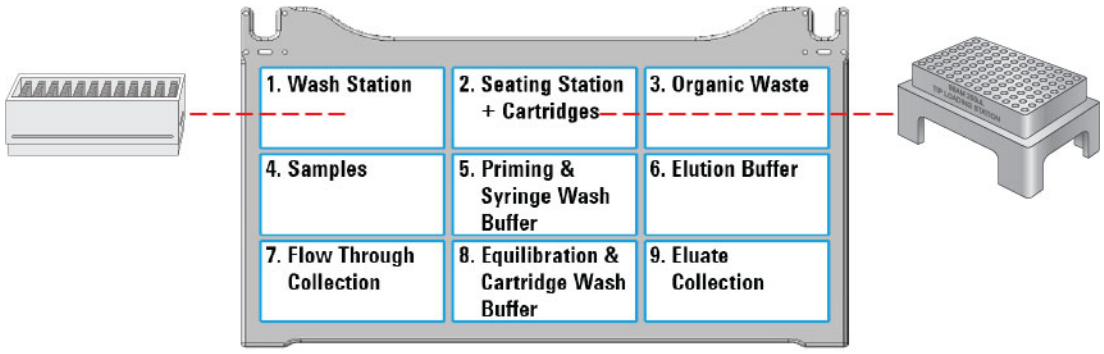
Labware

Labware requirements vary depending on experimental design. The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3, 5—8
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	3, 5—8
96 Agilent, 2mL Square Deep Well labware	Agilent 204353-100	3, 7

Labware	Manufacturer part number*	Deck location options
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	3—8
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7, 9
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7, 9**
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	3—9
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	3—9
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	3—9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	3—9
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	4, 7, 9
96 V11 Manual Fill Reservoir	Agilent G5498B#049	5, 6, 8

*For dimensionally equivalent alternatives and other labware details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**The Greiner PCR plate is not compatible with the 25 µL cartridges at deck locations 7 and 9.

Reagents

The volume, type, and concentration of reagents required for protein cleanup vary depending on sample characteristics and the desired analytical result. Consult the published scientific literature and "Reference library" on page 504 to find specific chemistry examples. See the Agilent AssayMAP Bravo Citation Index for published papers that use the AssayMAP Protein Cleanup application.

By default, the syringes are rinsed thoroughly with deionized water at the wash station after completing the protocol to reduce the risk of premature syringe failure. To perform more stringent syringe washing between runs, use the Syringe Wash utility. For details, see the Syringe Wash v3.0 User Guide.

All labware require volume overage for the protocol to execute properly. Use the Reagent Volume Calculator to determine volume requirements for specific protocol conditions. See "Preparing the solutions" on page 472.

Preparing the solutions

The following solutions are required for the Protein Cleanup protocol:

- Priming & Syringe Wash Buffer
- Equilibration & Cartridge Wash Buffer

- Elution Buffer

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Using the Reagent Volume Calculator for Protein Cleanup

The Reagent Volume Calculator is a Microsoft Excel file that contains the following:

- *Calculator worksheet*. You enter the number of columns to process, whether to perform the Collect Flow Through option, the volume for each step in the protocol, the number of wash cycles to conduct, and the labware selection for each deck location. The calculator determines the volumes required based on your input, taking into consideration pipetting overage and evaporation concerns.

  Note: The pipetting overage suggested is generally conservative. The minimal overage may be greater or less depending on the volatility of the solution, the length of the run, and when the step occurs during the run. The overage volume can be optimized to minimize loss of precious reagents.
- *Reagent Recipe worksheet*. You enter the concentrations of each component in your reagent, and the worksheet calculates the recipe volumes required.

To use the Reagent Volume Calculator:

1. Open the **App Library.**
2. Locate the application, and then click the corresponding **Calculator** button. Microsoft Excel starts and displays the calculator.
3. Ensure that you enable content in Microsoft Excel.
4. Click one of the following:
   - **Set defaults for 5µL cartridges**. Sets the values in the calculator using the values from the default method for the 5 µL cartridges.
   - **Set defaults for 25µL cartridges**. Sets the values in the calculator using the values from the default method for the 25 µL cartridges.
5. Modify the values in the green boxes as required to match your specific method. As you change the values in the green boxes, the calculated values change.

   Note: The green box should remain green after you enter a value. If you enter a value that is outside the normal working range, the box becomes yellow. If you enter a value that is outside of the acceptable range, the box becomes red.

   To display the corresponding tooltip for a setting, mouse over a box that has a red triangle in the upper right corner.

The following figures show the worksheets of the Reagent Volume Calculator.

Figure Protein Cleanup Calculator worksheet

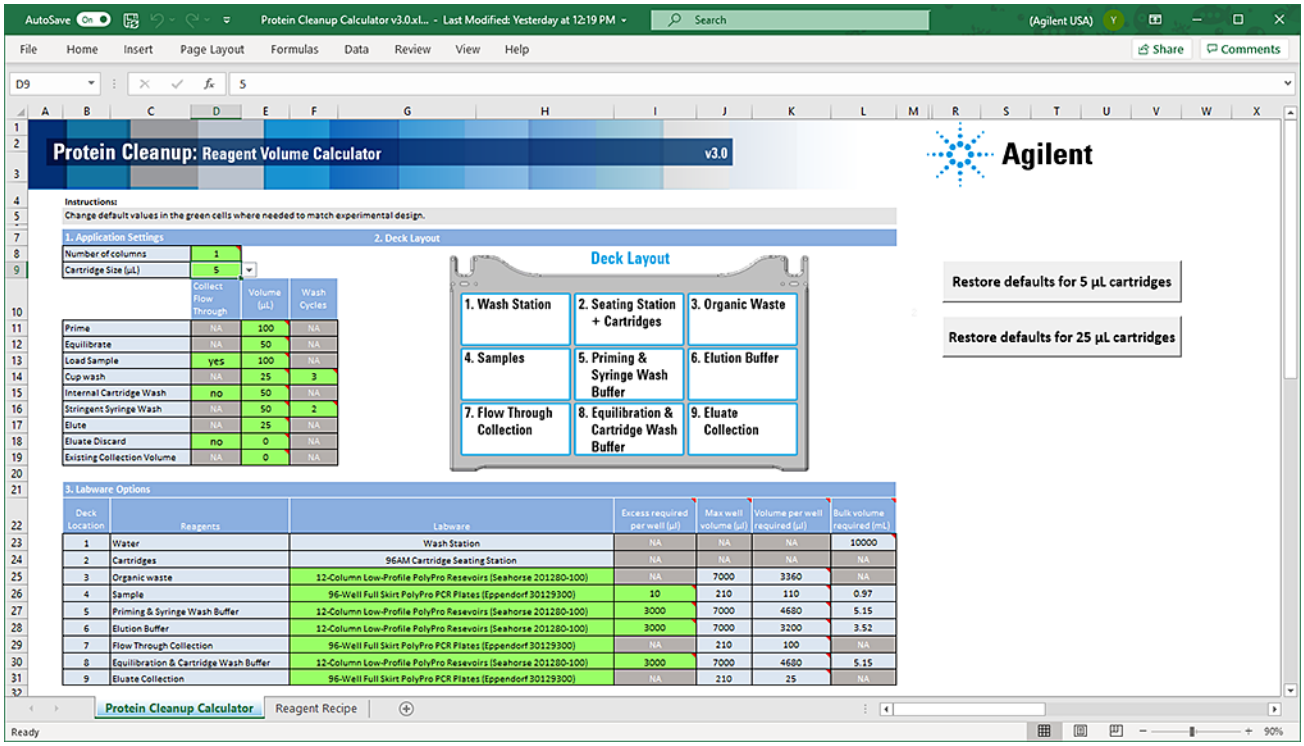
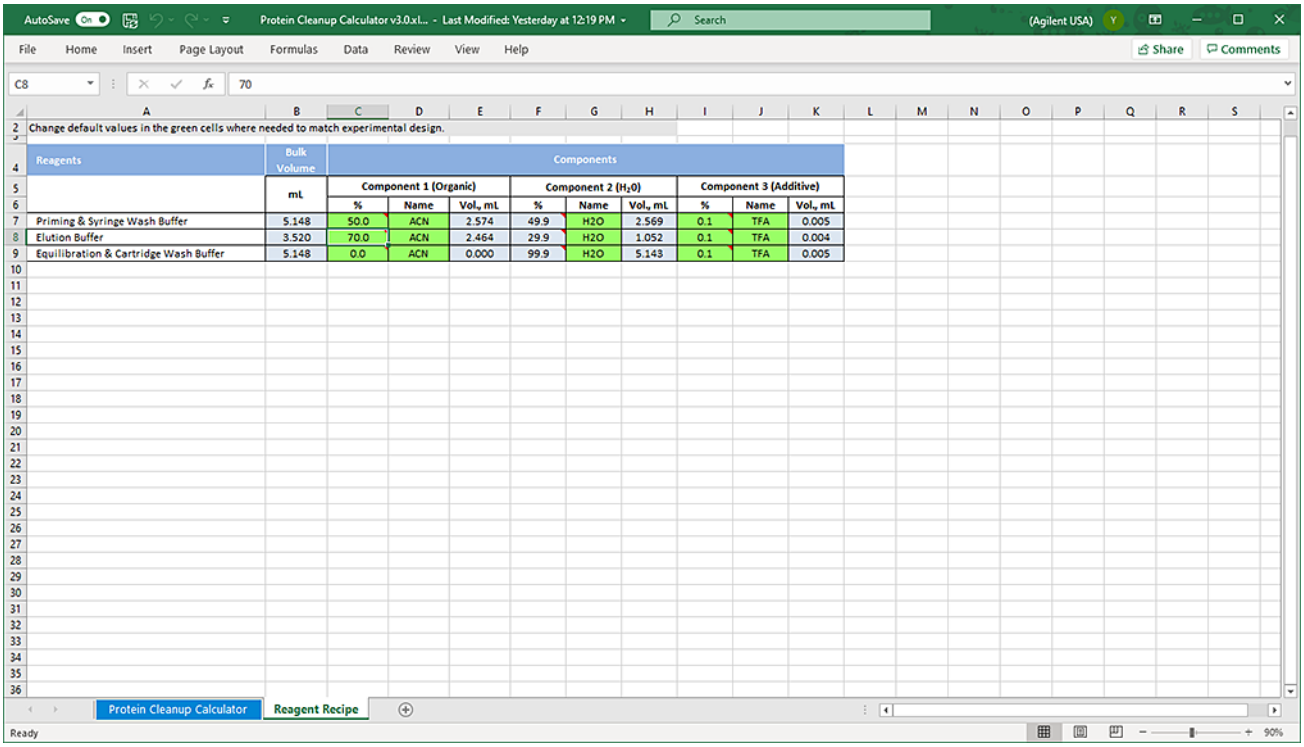


Figure Protein Cleanup Reagent Recipe worksheet



Preparing the solutions

The following table describes the reagents and deck locations. The AssayMAP protocols are blind to the composition of the solutions, so you can easily adapt your optimized chemistry. Agilent recommends the buffers listed in the following table as a **starting point** for optimizing the AssayMAP Protein Cleanup chemistry.

Table Reagent preparation

Reagent (deck location)	Composition and comments
Priming & Syringe Wash Buffer (deck location 5)	Typically 50% or greater organic (must be >25% organic), acidic, and identical in composition to the Elution Buffer to simplify the solution preparation, for example, 60% ACN: 39.9% H_2O : 0.1% TFA The high-percentage organic composition is essential for reversed-phase cartridges as they ship dry and must be wetted with a high percentage organic solution or they will have very little binding capacity. This solution, the other solutions used in this application, and the sample are typically acidic. Other more mass-spec-friendly acids can substitute for TFA.
Elution Buffer (deck location 6)	High percentage organic and acidic solution, for example, 60% ACN: 39.9% H_2O: 0.1% TFA *Note:* Carefully determine the percentage of organic solution and the pH as they both strongly influence the elution efficiency.
Equilibration & Cartridge Wash Buffer (deck location 8)	Very low or no organic and acidic solution. Typically, similar in the percentage organic and acidity to the sample, for example, 99.9% H_2O : 0.1% TFA

Dispensing the solutions

IMPORTANT To prevent evaporation, dispense the solutions into the labware immediately before running the protocol, or keep the plates lidded until the run begins.

IMPORTANT If you are using fewer than 96 cartridges, make sure you fill the labware to correspond with the sample layout in the sample plate and the cartridge positions in the 96AM Cartridge & Tip Seating Station. For more information, see "Preparing the samples" on page 476.

To dispense the solutions into the labware:

1 *Optional*. Label each piece of labware so that you can easily identify them.

2 Add the specified volume of Priming & Syringe Wash Buffer into the labware to be placed at deck location 5.

3 Add the specified volume of Elution Buffer into the labware to be placed at deck location 6.

4 Add the specified volume of Equilibration & Cartridge Wash Buffer into the labware to be placed at deck location 8.

5 If necessary, centrifuge the filled labware to remove bubbles.

Note: You can use the Reagent Aliquot utility to dispense the buffers. For details, see "Reagent Aliquot v2.0 User Guide" on page 518.

Preparing the samples

IMPORTANT

To minimize evaporation, prepare samples immediately before running the Protein Cleanup protocol, or keep the plates lidded until the run begins.

When preparing the samples:

- Remove macromolecular particulates before the samples are loaded onto AssayMAP cartridges.
- Adjust the buffer composition to the optimal binding conditions (for example, low pH and low organic).
- Determine the sample volume to load on the AssayMAP cartridges.
- Transfer the samples to the microplate you want to use for the protocol run.

Removing macromolecular particulate

Make sure the samples are free of macromolecular particulates, such as large protein aggregates and cellular debris to prevent clogging the cartridges. Samples should be filtered through a 0.45-µm filter or centrifuged at a high g-force immediately before loading on an AssayMAP cartridge.

Adjusting the buffer composition

The Protein Cleanup application can be used for a wide variety of proteins. Given the range of physiochemical properties that proteins display, you should examine the relevant scientific literature to determine the sample buffer conditions that favor efficient binding and avoid negatively affecting your protein of interest. In general, acidification, which can be accomplished by the addition of TFA, formic acid, or acetic acid to the sample, improves binding to reversed phase resins such as RP-W.

Agilent app note 5991-6478EN ("Reference library" on page 504) provides a detailed analysis of the optimal conditions for using the RP-W cartridges for the cleanup of denatured antibodies. This app note focuses on the cleanup of denatured antibodies to enable rapid trypsin digestion, but it also provides a general approach to optimize the conditions to clean up your protein of interest.

Avoid samples containing organic solvents or some types of detergents as they might inhibit binding to the cartridge. For example, loading samples in a buffer containing greater than 5% acetonitrile might inhibit binding of some proteins. If you have concerns about a specific buffer component, you should survey the scientific literature for any known effects of this type of molecule on reversed-phase resins.

The Reagent Transfer utility is a simple way to adjust the pH or dilute your sample before loading it onto the RP-W cartridge. For instructions, see "Reagent Transfer v3.0 User Guide" on page 525.

Note: A reverse-phase protein clean up may not work for all proteins due to limited solubility in the high organic solvent, which is required for elution. In addition, when desalting a denatured protein, the removal of the denaturation reagent may cause solubility problems.

Determining the volume of sample to load

The Protein Cleanup protocol permits loading of up to 1000-µL sample volume onto the AssayMAP cartridges. If the sample volume is greater than 250 µL, the protocol will iteratively load samples onto the cartridges to stay within the maximum AssayMAP syringe volume (250 µL).

What is the binding capacity of the cartridge?

Two ways to express the binding capacity of a cartridge are quantitative binding capacity and total binding capacity:

- *Quantitative binding capacity*. The maximum mass of protein that can bind to the cartridge in a single pass, where less than 10% of the load appears in the flow-through.

  The quantitative binding capacity depends on the composition of the solution in which the protein is bound. For denatured antibodies, the binding capacity is slightly reduced when there is greater than 1.5 M guanidine in the sample and if the sample is not acidified.
- *Total binding capacity*. The maximum mass of protein that can bind to the cartridge. This can only be achieved only by loading significantly more protein than can be bound by the cartridge. This value is significantly greater than the quantitative binding capacity.

See the Agilent AssayMAP Bravo Cartridges Selection Guide for detailed information about the binding capacity for the RP-W cartridges.

What is the concentration of the target in the sample?

If you know the approximate concentration of the target molecule in your sample and you are working within the quantitative binding range of the cartridge, you can determine the volume of sample to load as follows:

$$\text{µL sample to load} = \frac{\text{µg target desired}}{\text{µg/µL target in sample}}$$

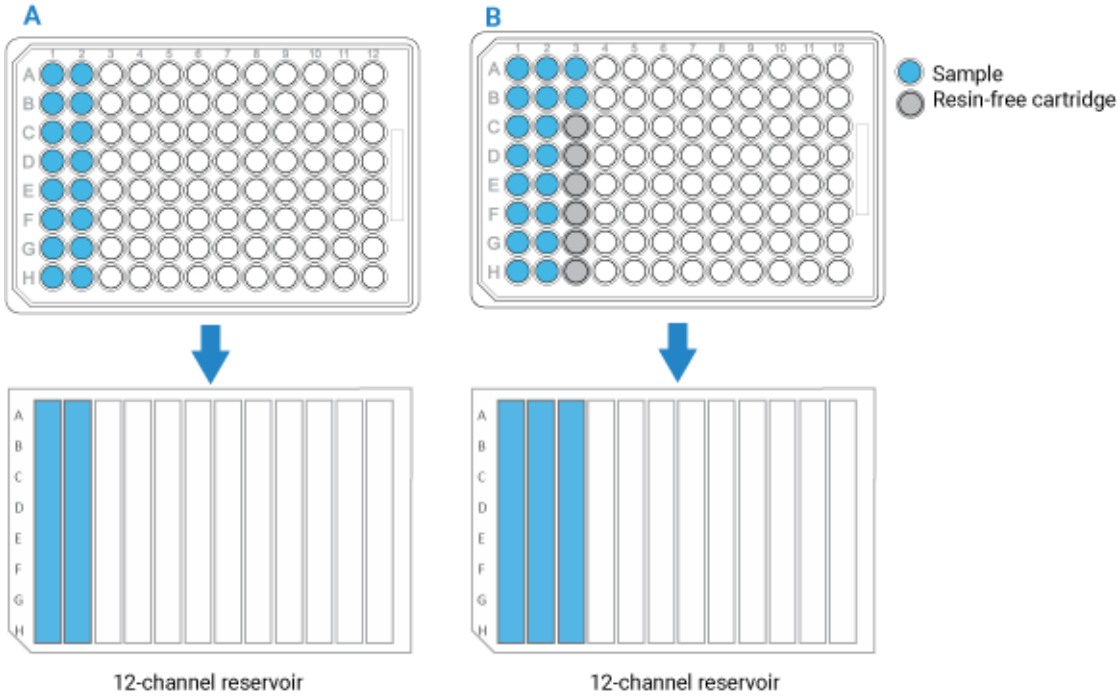
Preparing the sample plates

Planning the microplate setup

Before transferring the samples, you should plan the layout of the samples in the microplate. Consider the following:

- You can process 1 to 96 samples in parallel. The position of the samples in the microplate dictates the positions of the cartridges in the 96AM Cartridge & Tip Seating Station. These positions must match the locations of the buffer solutions in microplates and reservoirs.
- If you have fewer than 96 samples, make sure the samples occupy full columns in the microplate, as the following figure shows.
- The default protocol settings assume that samples will be arranged in multiples of 8 in a column-based configuration. Also, the AssayMAP Bravo Platform applies differential pressure to seat cartridges based upon the number of full columns of cartridges. To achieve proper cartridge seating, entire columns must be used.
- If the number of samples you have is not a multiple of 8, use the AssayMAP Resin-Free cartridges to fill the empty column and row positions. This will prevent liquids from dripping on the deck or being dispensed on the deck during the Cup Wash step.

Figure Example of sample microplate and reservoir layout: A) Multiple of 8 samples, B) Not a multiple of 8



See "Labware" on page 471 for acceptable labware at each deck location.

Transferring the samples to the microplate

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

An excess (overage) volume ensures that a microplate well or column does not fully deplete, which would result in aspiration of air into the syringes and then into the cartridges, compromising performance.

The Reagent Volume Calculator shows the recommended overage for the labware types being used and automatically includes recommended overages in the volume it recommends per well. See "Using the Reagent Volume Calculator for Protein Cleanup" on page 473.

Labware-specific overage recommendations are also presented in the *Labware Reference Guide*, which you can find in the Literature Library page of the Protein Sample Prep Workbench. More or less overage can be used depending on the volatility of the solution and the length of the run but the recommended overages are fine for most standard runs.

To transfer the samples to the microplate:

1. Run the Reagent Transfer utility or Reformatting utility to move samples into one of the labware options for deck location 4. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623
2. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Protein Cleanup protocol does the following:

- Washes the syringes.
- Primes and equilibrates the cartridges to prepare for sample loading.
- Loads the samples onto the cartridges.
- Removes non-specific binding molecules from the cartridges.
- Elutes the protein from the cartridges.

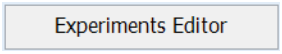
For some of these operations the cartridges are mounted on the syringe probes, while for other operations the cartridges are parked in the cartridge seating station.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

To create an experiment ID, you open the Experiments Editor by clicking



in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.

- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the buffers. See "Preparing the solutions" on page 472.
- Prepare the samples. See "Preparing the samples" on page 476.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup protocol to prepare the AssayMAP Bravo Platform for the run. See System Startup/Shutdown v3.0 User Guide.
- Transfer the cartridges to the 96AM Cartridge & Tip Seating Station. See "Cartridge Transfer v2.0 User Guide" on page 506.

IMPORTANT Cartridges ship dry and therefore contain air entrained in the resin bed. Failure to prime the cartridges can prevent the sample and buffers from accessing parts of the bed, resulting in reduced capacity and poor reproducibility.

IMPORTANT Do not allow wetted cartridges to dry out. Agilent Technologies does not guarantee performance of stored cartridges following equilibration. See "Cartridge use and storage guidelines" on page 470.

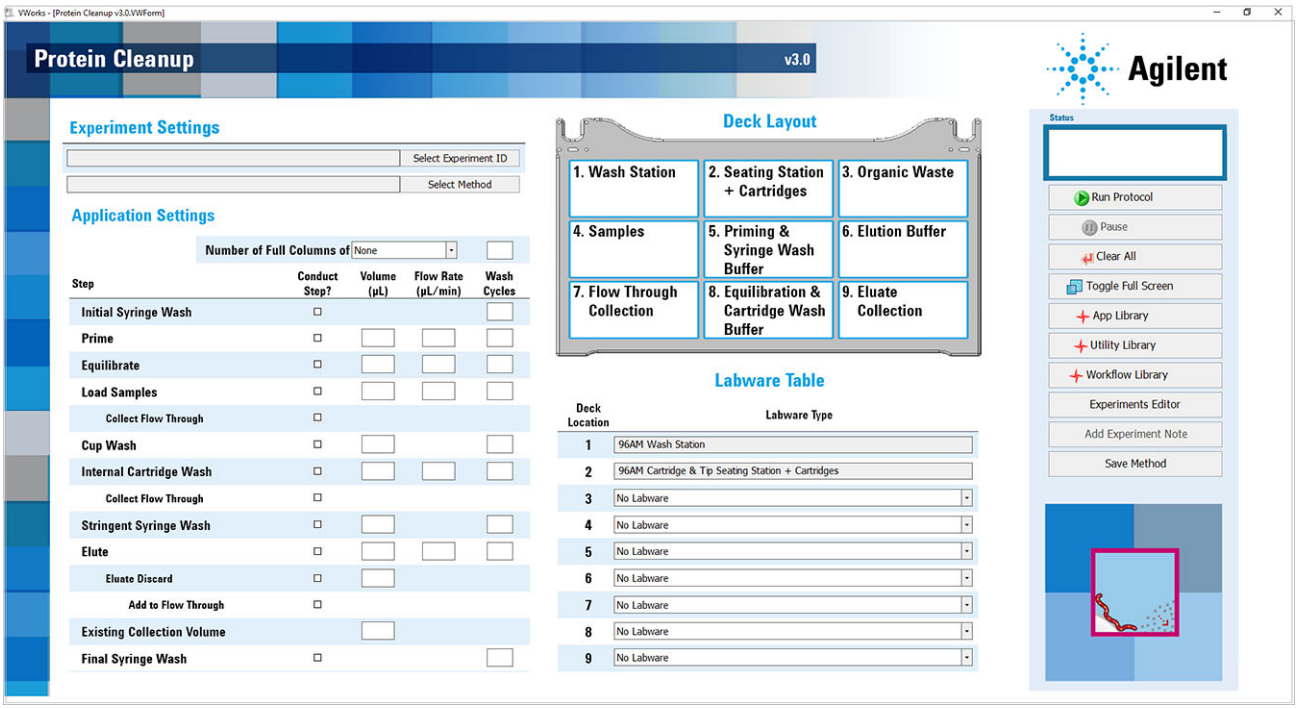
Setting up the protocol

Before starting the protocol, make sure the appropriate selections and values are specified in the Protein Cleanup application.

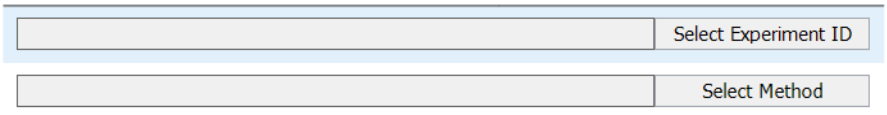
To set up the protocol:

1. Open the **App Library**.
2. Locate **Protein Cleanup v3.0**, and then click **App**.

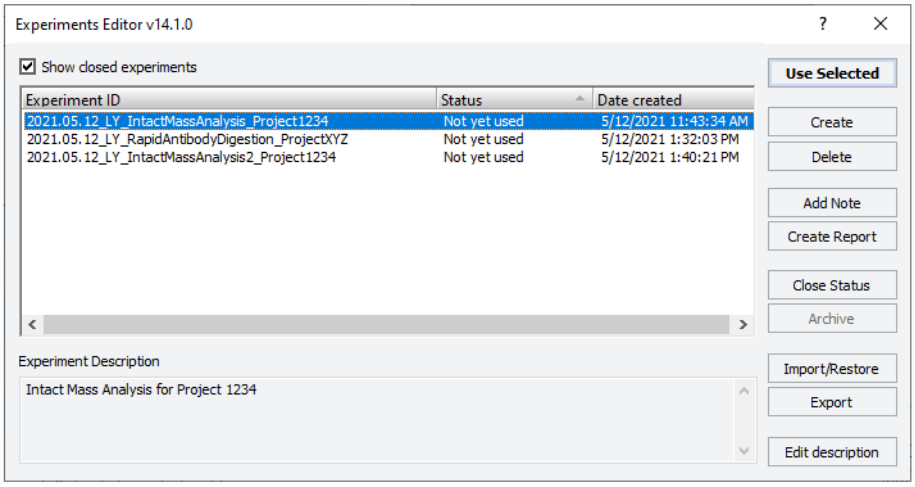
The Protein Cleanup application opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the protocol run" on page 485.
- To create or modify a method, proceed to step 6.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the cartridge settings:

a Select the cartridge size from the list:

- 5 µL Cartridges
- 25 µL Cartridges

b In the box, type the number of full columns of cartridges to be used.

The position of the columns of cartridges in the tip seating station must match the positions of the samples and solutions in the plates on the deck.

Range: 1–12

Default: 1

CAUTION

If the column selection is greater than the actual number of columns used, the Bravo Platform will apply too much force when mounting the cartridges, which can cause damage to both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. To prevent potential equipment damage, ensure that the column selection is correct.

CAUTION **If the column selection in the software is less than the actual number of cartridges used, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable. To obtain expected instrument performance, ensure that the column selection is correct.**

IMPORTANT Each full column must contain eight cartridges. If a column contains fewer than eight packed cartridges, use the AssayMAP Resin-Free cartridges to fill the empty column positions.

7 Under **Application Settings,** select the check boxes of the steps that you want to perform, and enter the values for the selected steps.

Note: For any unselected steps, ensure that the volume, flow rate, and wash cycles boxes are blank to avoid potential confusion when a experimental report is generated.

8 In the **Labware Table** area, select the labware you are using for the protocol run.

Note: If all the steps that use a certain labware location are unchecked, ensure that the labware selection is No labware to avoid confusion when setting up the deck and when generating an experimental report. The Reagent volume calculator is a good resource for this decision because it returns a value of zero in the Volume per well required cell if no labware is needed.

9 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

Note: Agilent recommends that you use the cartridge size (5 µL or 25 µL) as a prefix to the name.

VWorks Plus. You must save the method before you can run it.

Application Settings

The following table gives a brief description of each setting. For details, including the full and practical ranges of values for a given setting, see the "Assay development guidelines and protocol notes" on page 488.

Table Application Settings overview

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	Washes syringes at the wash station (deck location 1).	5 µL:	–	–	3
		25 µL:	–	–	3
		Range:	–	–	0–10
Prime	Aspirates Priming Buffer (deck location 5) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	100	300	1
		25 µL:	250	300	1
		Range:	0–250	0.5–500	0–10

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Equilibrate	Aspirates Equilibration Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3).	5 µL:	50	10	1
		25 µL:	250	10	1
		Range:	0–250	0.5–500	0–10
Load Samples	Aspirates samples (deck location 4) into the syringes, and then dispenses them through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 7).	5 µL:	100	5	3
		25 µL:	100	5	3
		Range:	0–1000	0.1–500	0–10
Collect Flow Through	If selected, collects the sample flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the sample flow-through into the Organic Waste plate (deck location 3).	–	–	–	–
Cup Wash	Rinses the cartridge cups with Cartridge Wash Buffer (deck location 8), and then discards the liquid into the Organic Waste plate (deck location 3).	5 µL:	25	–	3
		25 µL:	25	–	3
		Range:	0–100	–	0–10
Internal Cartridge Wash	Aspirates the Cartridge Wash Buffer (deck location 8) into the syringes, and then dispenses it through the cartridges into the Organic Waste plate (deck location 3) or into the Flow Through Collection plate (deck location 7).	5 µL:	50	10	3
		25 µL:	250	10	3
		Range:	0–250	0.5–500	0–10
Collect Flow Through	If selected, collects the Internal Cartridge Wash flow-through in the Flow Through Collection plate (deck location 7). If not selected, discards the Internal Cartridge Wash flow-through into the Organic Waste plate (deck location 3).	–	–	–	–
Stringent Syringe Wash	Aspirates the Syringe Wash Buffer (deck location 5) into the syringes, and then dispenses it into the Organic Waste plate (deck location 3).	5 µL:	50	–	2
		25 µL:	50	–	2
		Range:	0–250	–	0–10
Elute	Aspirates the Elution Buffer (deck location 6) into the syringes, and then dispenses it through the cartridges into the Eluate Collection plate (deck location 9).	5 µL:	25	5	1
		25 µL:	125	5	1
		Range:	0–250	0.1–500	0–10
Eluate Discard	If selected, a specified initial volume of the eluate is discarded into the Organic Waste plate (deck location 3), or collected in the Flow Through Collection plate (deck location 7).	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–250	–	–

Steps*	Description	Cartridge size	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Add to Flow Through	If selected, collects the Eluate Discard in the Flow Through Collection plate (deck location 7). If not selected, discards the Eluate Discard into the Organic Waste plate (deck location 3).	–	–	–	–
Existing Collection Volume	Specifies the volume of liquid present in the Eluate Collection plate (deck location 9) at the beginning of the run.	5 µL:	0	–	–
		25 µL:	0	–	–
		Range:	0–300	–	–
Final Syringe Wash	Washes the syringes at the wash station (deck location 1).	–	–	–	3
		–	–	–	3
		–	–	–	0–10

*For practical value ranges for the steps listed in this table and factors to consider when changing the default values, see "Protocol stepwise guidelines" on page 488.

For a complete list of the robotic movements executed during a run, see "Automation movements during the protocol" on page 500.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. To decrease the run time, you can increase the flow rates to 500 µL/min, change the wash cycles to 1, and decrease the volumes. Use the AssayMAP Resin-Free cartridges instead of packed cartridges for mock runs.

IMPORTANT

The protocol will display an error message if cartridges are missing.

Starting the protocol run

WARNING

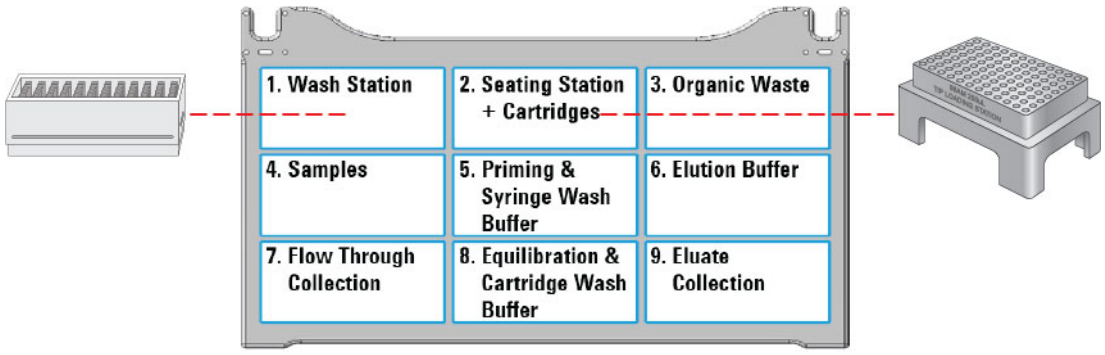
The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.

Note: The Greiner PCR plates are not compatible with the 25 µL cartridges at deck locations 7 and 9.

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

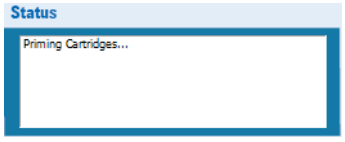
Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

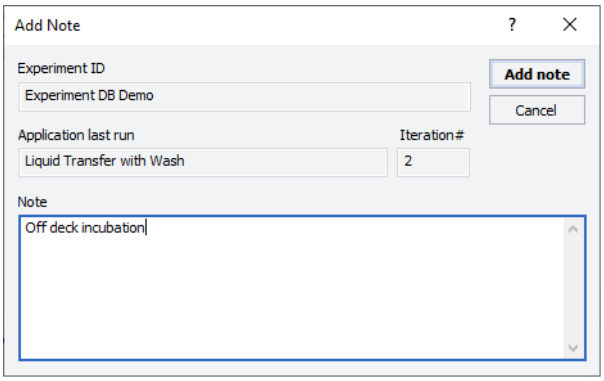
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

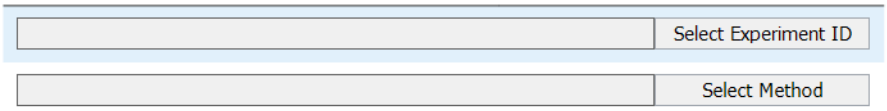
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click Run Protocol to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines and protocol notes

This topic explains the following:

- Each step of the protocol so that you can optimize the Protein Cleanup protocol to your particular experimental design
- Automation movements during the protocol

For details on how to use the Experiments Editor, see "Using Experiment IDs" on page 23.

Protocol stepwise guidelines

Protocol step	Guidelines and notes
Number of Full Columns of Cartridges	This setting is critical to set the proper force used to mount the cartridges. To obtain expected instrument performance, ensure that the column selection is correct. If the column selection is: • *Greater than the actual number of columns used*, the Bravo Platform will apply too much force when mounting the cartridges, which can damage both the cartridges and the AssayMAP syringes in the head. For example, if the software specifies 12 columns, but only 1 column of cartridges are in the seating station, the head will apply 12 times more force than what is required. • *Less than the actual number of columns used*, the Bravo Platform will not apply enough force to seat the cartridges properly. For example, if the software specifies 1 column, but 12 columns of cartridges are in the seating station, the head will apply 1/12th the force required to seat the cartridges properly. In this case, cartridges may fall off during the run or the volume of liquid that moves across the cartridge bed may be variable due to liquid moving past the syringe cartridges seal into the cartridge cup. Default: 1 Range: 1-12

Protocol step	Guidelines and notes
Initial Syringe Wash	This step removes any potential contaminants from the syringes at the wash station before the cartridges are mounted. During each Initial Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys and then moves by a fixed offset between the wash station chimneys to dispense to waste. This step is selected by default. **Wash Cycles.** Increasing the number of wash cycles may clean the syringes better. However, more cycles increases the total run time and causes wear on the syringes. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Prime	This step removes entrained air from the packed resin bed and properly wets the surface of the resin. In preparation for priming, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Priming Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Prime step aspirates the Priming Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. The AssayMAP reversed-phase cartridges (RP-W) used with the Protein Cleanup application require a Priming Buffer containing at least 25% organic solvent. As illustrated in Agilent app note 5991-6478EN ("Reference library" on page 504), reversed-phase cartridges (RP-W) that are primed with solutions containing a low percentage of organic solvent have greatly reduced binding capacity. Agilent recommends using a priming solution that contains at least 50% organic solvent, such as acetonitrile. This step is selected by default. **Volume (µL)**. The default volume is sufficient to wet and remove entrained air from the resin bed. Using less than the default volume may leave air in the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 100 – Practical: 100–250 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Prime tasks except syringe washing. **Flow rate (µL/min)**. A flow rate lower than the default value diminishes the ability to effectively remove entrained air from the cartridge. A flow rate faster than the default is not required and has not been tested. • Default: 300 • Practical: 300 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Equilibrate	This step ensures that the resin bed is fully equilibrated with a solution that provides the optimal chemical conditions for binding during the Load Samples step. In preparation for equilibration, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Equilibration Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. During the Equilibrate step, the Equilibration Buffer is aspirated into the syringes, the cartridges are mounted, and then the buffer is dispensed through the cartridges into the Organic Waste plate. The cartridges are parked at the seating station and the syringes are washed at the wash station. The Agilent reversed-phase cartridge (RP-W) typically used with the Protein Cleanup application require an equilibration solution that has a very low concentration of or no organic solvent for effective binding during the sample loading step. This step is selected by default. **Volume (µL)**. The default volume is equal to 10 column volumes, which should be sufficient for complete buffer exchange. Using less than the default volume may not fully equilibrate the resin bed. Using more than the default volume is unnecessary and increases run time. • Volume for 5 µL cartridge: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridge: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Equilibrate tasks except syringe washing. **Flow rate (µL/min)**. A flow rate slower than the default rate will likely have no benefit, but will increase the total assay time. A flow rate faster than 20 µL/min using the default volume may not equilibrate through the pores in the beads. • Default: 10 • Practical: 5–20 • Range: 0.5–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10

Protocol step	Guidelines and notes
Load Samples	This step allows the target analytes to bind to the surface chemistry of the resin bed. No liquid is removed or added to the cartridge cups before the sample loading begins. The assumption is that there is still liquid in the cups from the equilibration step that will prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. This step aspirates sample into the syringes, and then performs an external syringe wash at the wash station to remove any sample remaining on the outside of the probes before mounting the cartridges. The samples are dispensed through the cartridges into the Flow Through Collection plate or Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any sample on the exterior of the cartridges, the cartridges are parked at the seating station, and the syringes are washed at the wash station. The protocol accommodates sample volumes up to 1000 µL to be dispensed through the AssayMAP protein cleanup cartridges. Although, the form permits you to enter smaller volumes, the minimum advisable sample volume to be loaded onto an AssayMAP cartridge is 10 µL. Each syringe has a maximum capacity of 250 µL. When sample volumes are greater than 250 µL, the protocol will iteratively load volumes onto the cartridges. To determine the number and volume of iterative load steps, the protocol uses the following formulas: • *# of times to load* = total sample volume/250, where # times to load is rounded up to nearest integer • *volume of each load* = sample volume/# of times to load For example, if the total sample volume is 900 µL, then: *# times to load* = 900/250 = 3.6, which is rounded up to 4 *volume of each load* = 900/4 = 225 If Collect Flow Through is selected for the Load Samples step, be sure that the Flow Through Collection plate has sufficient maximum well capacity. For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. **IMPORTANT** Be sure to include the recommended labware-specific volume overage to prevent air from entering the cartridge. For more information, see "Transferring the samples to the microplate" on page 478. This step is selected by default.

Protocol step	Guidelines and notes
	Volume (µL): The volume of sample should be balanced with the sample concentration and the mass capacity of the cartridge. Large sample volumes (> 250 µL) may require slightly more excess sample due to evaporation. • Default: 100 • Practical: 10–1000 • Range: 0–1000 *Note:* The lower the sample volume, the higher the percentage of the total volume is overage. To minimize sample loss, Agilent recommends diluting small volume samples. *Note:* Setting the volume to zero skips all Load Samples tasks except syringe washing. **Flow rate (µL/min)**. The optimum sample loading flow rate requires balancing the speed of the assay and desired recovery. When setting the flow rate, be aware that the quantitative binding capacity is inversely proportional to the flow rate. Therefore, the maximum possible quantitative binding capacity is only obtained with very slow sample loading flow rates. If the amount of sample that you want to capture is significantly lower than the total possible qualitative binding capacity, you will be able to use a faster flow rate while maintaining quantitative binding. Using flow rates slower than the default may not significantly increase analyte binding, and using flow rates faster than the default will decrease the quantitative binding capacity of the cartridges. • Default: 5 • Practical: – 2–10 (5 µL cartridges) – 5–20 (25 µL cartridges) • Range: 0.1–500 **Wash cycles**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 • Range: 0–10
Collect Flow Through	If this step is selected, the sample flow-through from the Load Samples step is dispensed in the Flow Through Collection plate. If this step is not selected, the flow-through from the Load Samples step dispensed in the Organic Waste plate. The Collect Flow Through step is skipped if the Load Samples step is not conducted. This step is selected by default.

Protocol step	Guidelines and notes
Cup Wash	This step removes the residual sample liquid that may remain above the resin bed after sample loading. The Cup Wash step aspirates Cartridge Wash Buffer into the syringes and then dispenses it into the cups of the parked cartridges. This liquid plus any residual liquid from the samples is aspirated from the cartridge cups. The protocol ensures that no cartridges are stuck to the probes before dispensing the liquid into the Organic Waste plate, and then washing the syringes at the wash station. This step is selected by default. **Volume (µL)**. Using a volume less than the default may be insufficient for cup washing, while using a volume >50 µL may offer little benefit. • Default: 25 • Practical: 25–50 • Range: 0–100 *Note:* Setting the volume to zero skips all Cup Wash tasks. **Wash cycle**. Each cycle comprises one cup wash and one syringe wash. • Default: 3 • Practical: 3–5 • Range: 0–10

Protocol step	Guidelines and notes
Internal Cartridge Wash	This step uses Cartridge Wash Buffer to wash non-specifically bound molecules from the resin bed. In preparation for the Internal Cartridge Wash, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Cartridge Wash Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. For the wash operation, this step aspirates the Cartridge Wash Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Flow Through Collection plate or Organic Waste plate. The exterior of the cartridge tips are washed at the wash station to remove any remaining buffer on the cartridge exterior, the cartridges are parked at the seating station, and the syringes are washed at the wash station. If the Load Samples step is selected, the first 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) of Cartridge Wash Buffer is dispensed as a sample chase at the Load Samples flow rate. Next, the Internal Cartridge Wash volume minus the chase volume is dispensed at the Internal Cartridge Wash flow rate. The sample chase ensures that the sample volume in the cartridges at the end of the sample load moves through the cartridge bed at the same rate as the rest of the sample. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume (10 column volumes) may improve the purification marginally but also increases the run time. Volumes lower than the default volume may be insufficient for efficient cartridge washing. • Volume for 5 µL cartridges: – Default: 50 – Practical: 50–100 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 250 – Practical: 250 – Range: 0–250 *Note:* Setting the volume to zero skips all Internal Cartridge Wash tasks except for syringe washing. **Flow rate (µL/min)**. A rate slower than the default flow rate will likely have little benefit, but will increase the total assay time. A rate faster than 20 µL/min may not equilibrate through the pores in the beads, resulting in incomplete washing. • Default: 10 • Practical: 5–20 • Range: 0.5–500

Protocol step	Guidelines and notes
Internal Cartridge Wash (continued)	**Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 3 • Practical: 2–5 Range: 0–10
Collect Flow Through	If this step is selected, the flow-through from Internal Cartridge Wash step is dispensed into the Flow Through Collection plate. If the Collect Flow Through step is not selected, the flow-through from the Internal Cartridge Wash step is dispensed into the Organic Waste plate. This step is not selected by default.
Stringent Syringe Wash	This step cleans the syringes with the Syringe Wash Buffer prior to elution. The Stringent Syringe Wash step aspirates the Syringe Wash Buffer into the syringes, draws the buffer through a full syringe stroke to ensure the entire syringe is rinsed, and then dispenses the buffer into the Organic Waste plate. The syringes are then washed at the wash station. This step is selected by default. **Volume (µL)**. Volumes higher than the default volume are unlikely to improve the syringe cleaning but will increase the reagent consumption. Volumes lower than the default volume may be insufficient for efficient syringe washing. • Default: 50 • Practical: 50–100 • Range: 0–250 *Note:* Setting the volume to zero skips all Stringent Syringe Wash tasks. **Wash cycle**. A wash cycle is a stringent syringe wash followed by a basic syringe wash at the wash station. • Default: 2 • Practical: 2–5 • Range: 0–10

Protocol step	Guidelines and notes
Elute	This step uses Elution Buffer to elute bound proteins from the cartridges. In preparation for elution, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate, 10 µL of Elution Buffer is aspirated into the syringes and then dispensed into the cartridge cups to prevent potential air gaps from being introduced when the cartridges are seated on the syringe probes. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the buffer through the cartridges into the Eluate Collection plate. An external cartridge tip wash is performed at the wash station to remove any sample on the outside of the cartridges, and then the cartridges are parked at the seating station. After the elution, the samples are mixed in the Eluate Collection plate and the syringes are washed at the wash station. *Note:* If the total volume in the Eluate Collection plate is <15 µL, the samples will not be mixed. You can also select the Eluate Discard and Add to Flow Through substeps, which are described in the following rows of this table. This step is selected by default. **Volume (µL)**: The volume of Elution Buffer required for complete elution of bound analyte from the resin bed is dependent on the strength of the Elution Buffer. So the minimum elution volume must be determined empirically. If a strong Elution Buffer is used, the minimum volume is approximately 2–3 column volumes (10–15 µL for 5 µL cartridges, or 50–75 µL for 25 µL cartridges). The default volumes are conservative and significantly higher than the minimum expected with a strong Elution Buffer. *Note:* The Eluate Collection plate must be able to accommodate the total volume, which is determined by summing the net elution volume (Elute volume - Eluate Discard volume) and the Existing Collection Volume. For labware-specific maximum well volumes, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. • Volume for 5 µL cartridges: – Default: 25 – Practical: 10–30 – Range: 0–250 • Volume for 25 µL cartridges: – Default: 125 – Practical: 50–150 – Range: 0–250 *Note:* Setting the volume to zero skips all Elute tasks except syringe washing.

Protocol step	Guidelines and notes
	Flow rate (µL/min). A flow rate slower than the default is unlikely to improve the elution yield. Elution yield may be compromised if flow rates are faster than 15 µL/min for a given volume of elution buffer (that is, more elution buffer may be required to get the same elution yield at high elution flow rates relative to using lower flow rates for a given elution volume). • Default: 5 • Practical: 5–15 • Range: 0.1–500 **Wash cycle**. The number of syringe wash cycles to perform at the end of this step. 250 µL of DI water is used for each syringe wash cycle. • Default: 1 • Practical: 1–3 • Range: 0–10
Eluate Discard	This substep of the Elute step permits a specified volume of the eluate from the cartridges to be discarded before the eluate starts to be collected during the Elute step. The Elute step aspirates the Elution Buffer into the syringes, mounts the cartridges, and then dispenses the Elution Buffer at the Elute flow rate through the cartridges. If the Eluate Discard step is selected, the specified volume is dispensed into the Organic Waste plate or Flow Through Collection plate (if the Add to Flow Through step is selected). The remaining Elution Buffer is dispensed through cartridges at the Elute flow rate into the Eluate Collection plate. **Example**: If the Elute, Eluate Discard, and Add to Flow Through steps are all selected with the following settings: Elute volume = 15 µL (5 µL cartridges) or 40 µL (25 µL cartridges) Eluate Discard volume = 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) the first 2 µL (5 µL cartridges) or 10 µL (25 µL cartridges) eluate from the cartridges will be discarded into the Flow Through Collection plate, and the remaining 13 µL (5 µL cartridges) or 30 µL (25 µL cartridges) eluate will be collected in the Eluate Collection plate. Select the Eluate Discard step in situations where minimizing the volume of eluate is important. For AssayMAP cartridges, the initial elution volume (~2 µL for 5 µL cartridges or ~10 µL for the 25 µL cartridges) contains small or no measurable amounts of analyte. This step is not selected by default. **Volume (µL).** The first volume of eluate that will be discarded during the Elute step. This value can equal, but cannot exceed the Elute volume. • Default: 0 • Practical: – 5 µL cartridges: 0–2 – 25 µL cartridges: 0–10 • Range: 0–250

Protocol step	Guidelines and notes
Add to Flow Through	If selected, this step dispenses the Eluate Discard volume into the Flow Through Collection plate. If the Add to Flow Through step is not selected, the Eluate Discard is dispensed into the Organic Waste plate. This step is not selected by default. *Note:* The Add to Flow Through step is an option only if both the Elute and Eluate Discard steps have been selected.
Existing Collection Volume	This step enables users to specify an amount of liquid that is in the wells of the Eluate Collection plate at the beginning of the run. The Existing Collection Volume and the net volume from the Elute step (Elute Volume - Eluate Discard Volume) feeds into logic that adjusts the well-bottom offset for sample elution, calculates the eluate mixing volume, and dynamically moves the head in and out of the wells during elution and eluate mixing in a volume-dependent manner. For the maximum practical working volumes of labware for eluate collection, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench. Select this step when the Eluate Collection plate contains a volume of liquid useful for immediately diluting the eluates, for adjusting the pH of the eluates, or to aid in the recovery of small volumes of eluates from AssayMAP cartridges. **Volume (µL):** • Default: 0 • Practical: 0—250 • Range: 0–300
Final Syringe Wash	This step uses the wash station to flush potential contaminants from the syringes. Before the final syringe wash begins, 20 µL of air is aspirated into the syringes, the probes go into the cartridge cups to a depth that is just short of the normal engagement position, liquid in the cups is removed by a 60 µL aspiration and then discarded into the Organic Waste plate. No solution is added into the cartridge cups. *Note:* If the Final Syringe Wash is skipped, the 10 µL of elution buffer will remain in the cartridge cups. During each Final Syringe Wash cycle, the head aspirates 250 µL into the syringes from the wash station chimneys, and then moves by a fixed offset between the chimneys to dispense the syringe contents to waste. *Note:* In cases where carryover is a major concern, increasing the number of wash cycles may provide improved washout, but with a cost of increased assay time and reduced syringe lifetime. The best practice is to use the Syringe Wash utility to wash the syringes between runs with stringent wash solutions. This step is selected by default. **Wash Cycles:** • Default: 3 • Practical: 3–5 • Range: 0–10

Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Protein Cleanup protocol using the default method. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
Starting protocol	2	Parks all cartridges that might have been loaded on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
Initial Syringe Wash	1	Washes the syringes.
Prime	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	5	Aspirates 10 µL of Priming Buffer for the cartridge air-gap prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	5	Aspirates the Priming Buffer.
	2	Mounts the cartridges onto the head.
	3	Dispenses the buffer through the cartridges into the Organic Waste plate to prime the cartridges.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Equilibrate	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	8	Aspirates 10 µL of Equilibration Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Equilibration Buffer.
	2	Mounts the cartridges on the head.
	3	Dispenses the Equilibration Buffer through the cartridges to equilibrate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Load Samples	4	Aspirates the samples into the syringes.
	1	Washes the exterior of the syringe probes.
	2	Mounts the cartridges on the head.
	7	Dispenses the samples through the cartridges and into the Flow Through Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Cup Wash	8	Aspirates the Cartridge Wash Buffer.
	2	Washes the cartridge cups and exercises the cartridges off task.
	3	Dispenses the Cartridge Wash Buffer into the Organic Waste plate.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Internal Cartridge Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	8	Aspirates 10 µL of Cartridge Wash Buffer for the cartridge air-gap prevention step.
	2	Dispenses the 10 µL of buffer into the cartridge cups and exercises the cartridges off task.
	8	Aspirates the Cartridge Wash Buffer into the syringes for the sample chase and the Internal Cartridge Wash steps.
	2	Mounts the cartridges on the head.
	3	Dispenses 5 µL (5 µL cartridges) or 25 µL (25 µL cartridges) Cartridge Wash Buffer through the cartridges at the Load Samples flow rate for the sample chase step.
	3	Dispenses the remaining Cartridge Wash buffer through the cartridges at the Internal Cartridge Wash flow rate and into the Organic Waste plate.
	2	Parks the cartridges in the seating station.
	1	Washes the syringes.
Stringent Syringe Wash	5	Aspirates the Priming & Syringe Wash Buffer.
	3	Dispenses the buffer.
	1	Washes the syringes.

Protocol step	Head moves to deck location...	Action
Elute	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the exterior of the syringe probes.
	6	Aspirates 10 µL of Elution Buffer for the cartridge air-gap-prevention step.
	2	Dispenses the 10 µL of Elution Buffer into the cartridge cups and exercises the cartridges off task.
	6	Aspirates the Elution Buffer.
	2	Mounts the cartridges on the head.
	9	Elutes the samples into the Elution Collection plate.
	1	Washes the exterior of the cartridge tips.
	2	Parks the cartridges in the seating station.
	9	Mixes the eluates.
	1	Washes the syringes.
Final Syringe Wash	2	Aspirates 20 µL of air above this location, moves down to just above the cartridge engagement point and aspirates 60 µL, and then exercises the cartridges off task.
	3	Dispenses into the Organic Waste plate.
	1	Washes the syringes.

Reference library

1 Bovee, M., Krahenbuhl, A. & Murphy, S., Rapid Antibody Digestion Enabled by Automated Reversed-Phase Desalting on the Agilent AssayMAP Bravo Platform, Agilent Application Note 5991-6478EN, 2016

See the Agilent AssayMAP Bravo Citation Index for published papers that used the AssayMAP Bravo Platform.

15 Utilities User Guides

Normalization, Reformatting and Serial Dilution utilities


"Normalization v3.0 User Guide" on page 585


"Reformatting v3.0 User Guide" on page 623


"Serial Dilution v3.0 User Guide" on page 653

Other utilities


"Cartridge Transfer v2.0 User Guide" on page 506


"Pipette Tip Transfer v2.0 User Guide" on page 512


"Reagent Aliquot v2.0 User Guide" on page 518


"Reagent Transfer v3.0 User Guide" on page 525


"Single Liquid Addition v2.0 User Guide" on page 542


"Syringe Test v2.0 User Guide" on page 549


"Syringe Wash v3.0 User Guide" on page 567

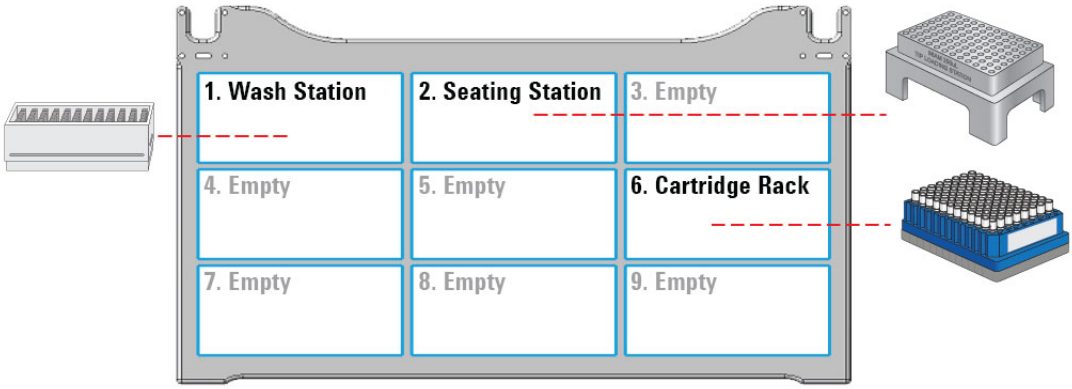

"System Startup/Shutdown v3.0 User Guide" on page 574

Cartridge Transfer v2.0 User Guide

The Cartridge Transfer utility moves full columns of AssayMAP Cartridges from the source cartridge rack, which is a standard 96AM Cartridge Rack and Receiver Plate at deck location 6, to the destination cartridge rack, which is the 96AM Cartridge and Tip Seating Station at deck location 2.

Before you start

The following figure shows the required deck layout.



On the AssayMAP Bravo deck, make sure:

- The empty seating station is at deck location 2.
- The 96AM Cartridge Rack and Receiver Plate is at deck location 6. It may be completely or partially filled with cartridges. Make sure you remove the lid.
- The cartridges are arranged in full columns of eight cartridges each (if the source 96AM Cartridge Rack and Receiver Plate is partially filled).
- The 96AM Wash Station or the later model 96 Channel Wash Station is at deck location 1.
- All other deck locations are empty.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.

- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

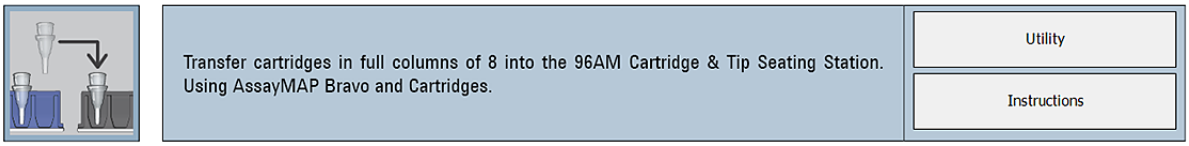
VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Setting up the protocol

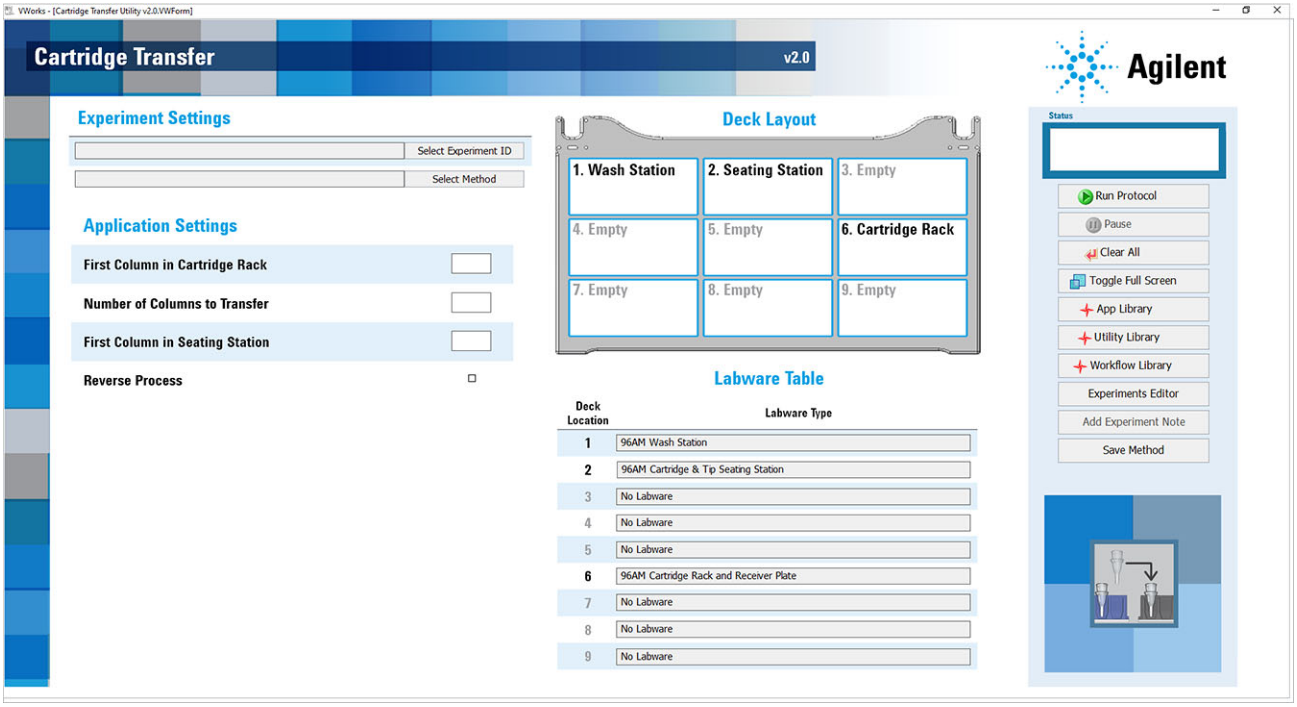
Before starting the protocol, make sure the appropriate selections and values are specified in the Cartridge Transfer utility.

To set up the protocol:

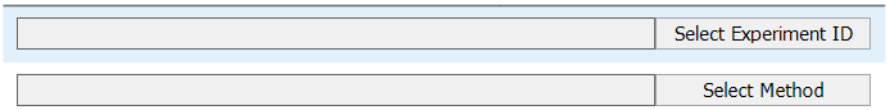
1. Open the **Utility Library**.
2. Locate **Cartridge Transfer**, and then click **Utility**.



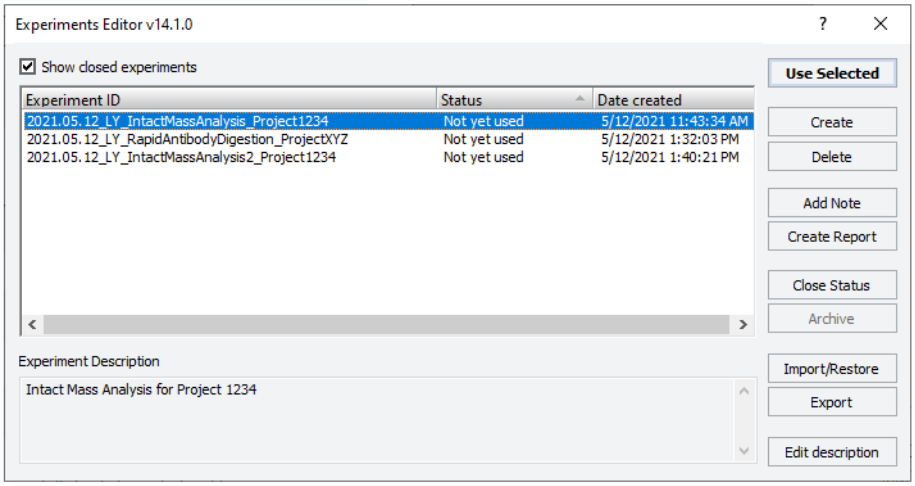
The Cartridge Transfer utility opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Cartridge Transfer run" on page 509.
- To create or modify a method, proceed to step 6.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the following settings for the run:

Setting	Description
First Column in Cartridge Rack	The first available column, counting from the left side of the 96AM Cartridge Rack and Receiver Plate. The value must be between 1 and 12.
Number of Columns to Transfer	The number of columns of cartridges that you want to transfer to the seating station. The value must be between 1 and 12.
First Column in Seating Station	The first column, counting from the left side, of the seating station to which cartridges will be transfered.
Reverse Process	The option to return the moved cartridges back to their original locations. See "Returning the cartridges to their original locations" on page 510.

7 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

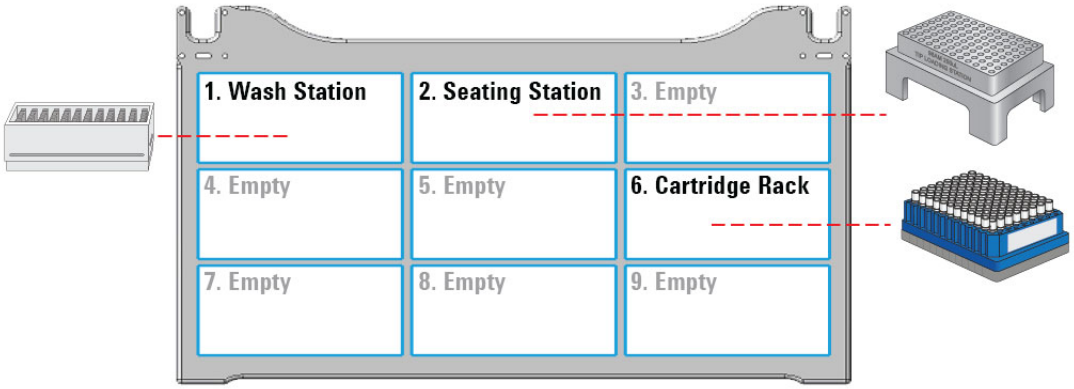
Starting the Cartridge Transfer run

WARNING

The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.

To start the protocol run:

1 Ensure that the accessories and cartridge rack are at the assigned deck locations, as shown in the **Deck Layout** image of the form.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box. For a summary of the movements, see "Automation movements during the Cartridge Transfer protocol" on page 511.

When the run is finished, remove the 96AM Cartridge Rack and Receiver Plate with any unused cartridges from deck location 6.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Returning the cartridges to their original locations

If you have accidentally transferred cartridges to the wrong columns of the seating station, you can return the cartridges to their original locations in the 96AM Cartridge Rack and Receiver Plate.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

To return the cartridges to their original locations:

1. Without changing any other settings from their original transfer process, select the **Reverse Process** check box.

   VWorks Plus. You must save the method before you can run it.

2. To save the Reverse Process method:

   a. Click Save Method.

   b. In the **Save File As** dialog box, type the file name and click **Save**.

3. In the form, click **Select Method.**

   In the **Open File** dialog box, select the Reverse Process method that you created in step 2, and click **Open**.

4. Click Run Protocol to reverse the original cartridge transfer process.

Automation movements during the Cartridge Transfer protocol

This section describes the basic movements of the AssayMAP Bravo Platform during a generic forward Cartridge Transfer protocol. Changing the selections or parameters will alter the movements.

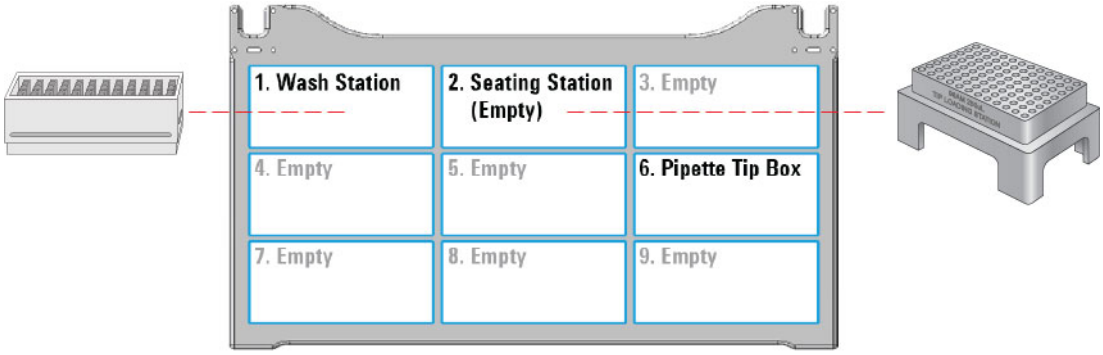
Protocol step	Head moves to deck location...	Action
1	6	Loads the desired number of cartridges.
2	2	Parks the cartridges in the seating station.

Pipette Tip Transfer v2.0 User Guide

The Pipette Tip Transfer utility moves 1–12 columns of Agilent 250-µL pipette tips from a source tip box at AssayMAP Bravo deck location 6 into an empty 96AM Cartridge & Tip Seating Station at deck location 2. This utility is designed to transfer contiguous, full columns of tips, where each column has eight pipette tips.

Before you start

The following figure shows the required deck layout.



On the AssayMAP Bravo deck, make sure:

- The empty seating station is at deck location 2.
- An Agilent 250-µL tip box is properly positioned at deck location 6. The tip box may be full or partially filled with pipette tips.

  For partially filled tip boxes, ensure that the tips are arranged in contiguous, full columns of eight pipette tips each.

  Make sure that the tip box lid has been removed.
- The 96AM Wash Station or the later model 96 Channel Wash Station is at deck location 1.
- All other deck locations are empty.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.

- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

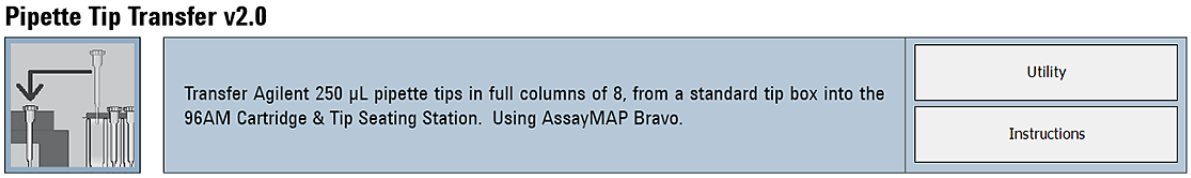
VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Setting up the protocol

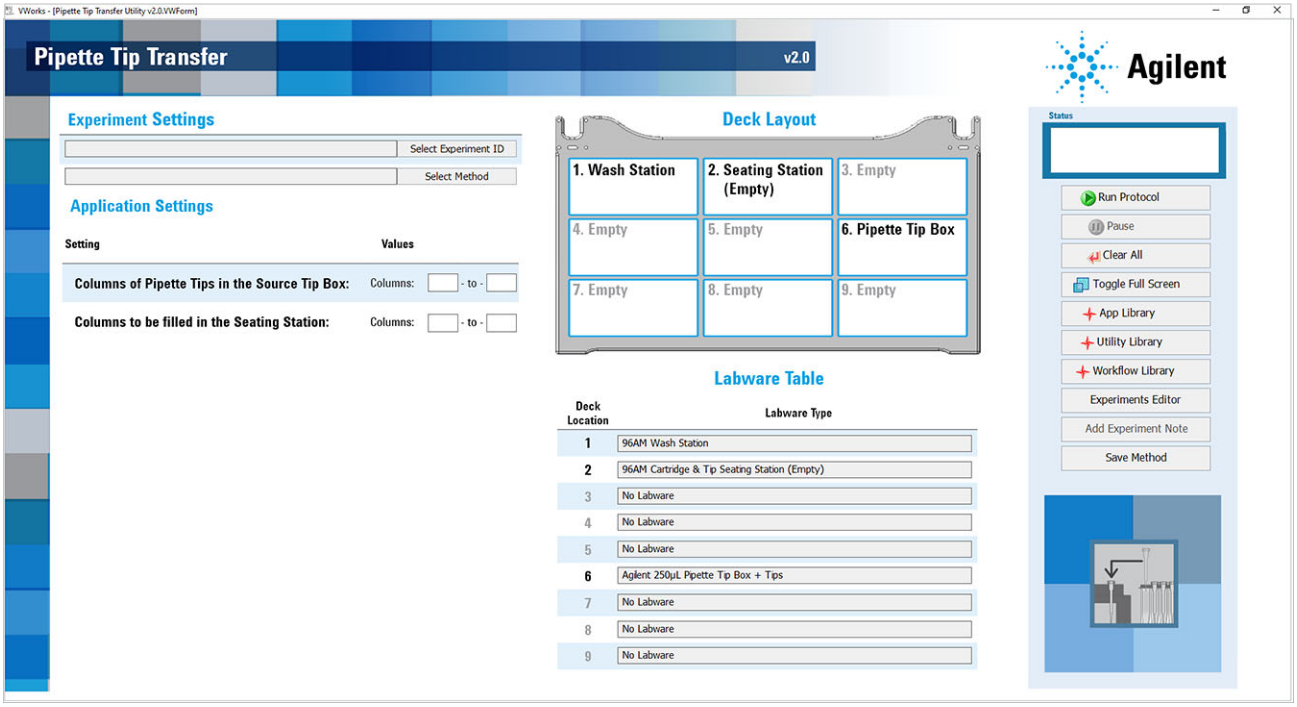
Before starting the protocol, make sure the appropriate selections and values are specified in the Pipette Tip Transfer utility.

To set up the protocol:

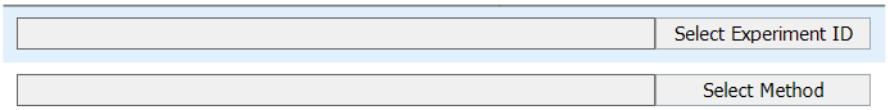
1. Open the **Utility Library**.
2. Locate **Pipette Tip Transfer**, and then click **Utility**.



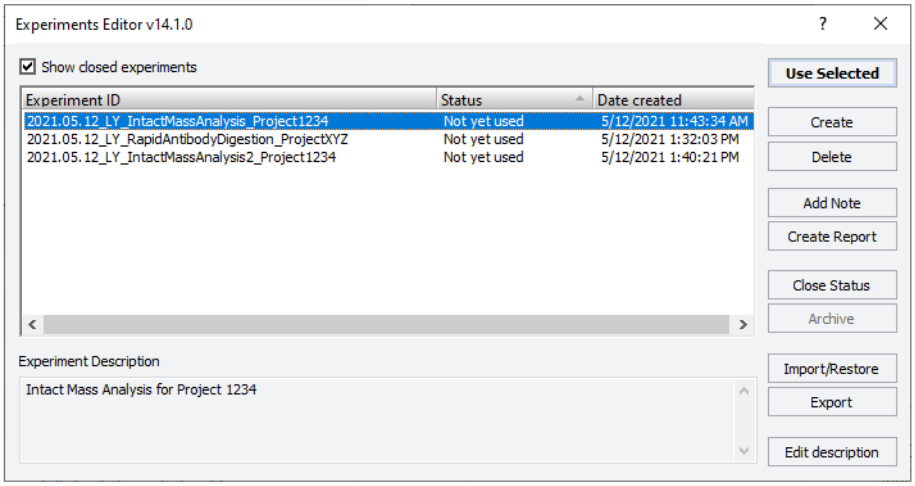
The Pipette Tip Transfer utility opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

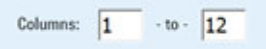
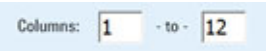
5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

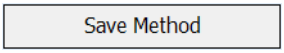
- To run the selected method, go to "Starting the Pipette Tip Transfer run" on page 515.
- To create or modify a method, proceed to step 6.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the following settings for the run:

Setting	Description
Columns of pipette tips in the Source Tip Box	Specifies the range of pipette tips initially present in the pipette tip box before the run starts.  • Left field is the first column that contains tips. • Right field: is the last column that contains tips. For example: • For a full tip box: left field = 1, right field = 12 • For partially filled tip box with tips in columns 6 - 10: left field = 6, right field = 10 **IMPORTANT** The pipette tip box must contain contiguous columns of 8 pipette tips each, within the range of columns specified.
Columns to be filled in the Seating Station	Specifies the range of wells to be filled with tips in the seating station at deck location 2.  • In the left field, specify the column number of the first column in the seating station that will contain tips after completing the transfer. • In the right field, specify the column number of the last column in the seating station that will contain tips after completing the transfer.

7 To save the method:

a Click  .

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

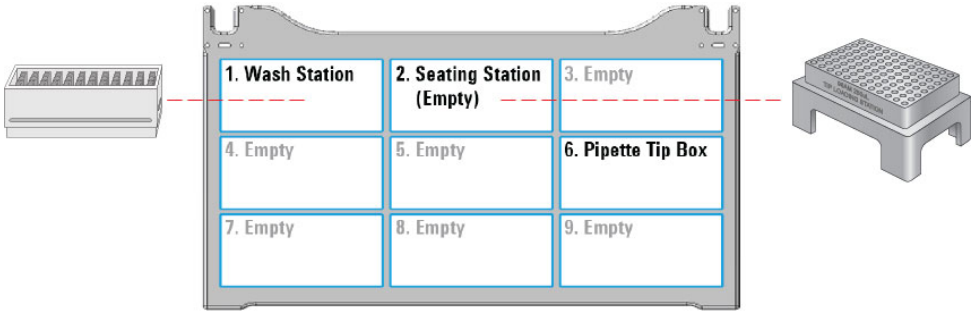
Starting the Pipette Tip Transfer run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories and pipette tip box are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

Make sure the labware are properly seated on the Bravo deck.

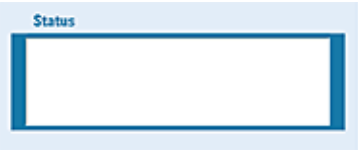


CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

CAUTION **A collision can occur if any pipette tips are present in the columns outside the specified range.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box. For a summary of the movements, see "Automation movements during the Pipette Tip Transfer protocol" on page 517.



When the run is finished, remove the Pipette Tip Box from deck location 6 from the deck.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

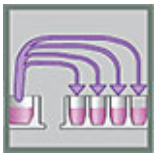
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Automation movements during the Pipette Tip Transfer protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Pipette Tip Transfer protocol using the Default Method settings. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
1	1	Dispenses any liquid remaining in the syringes into the wash station.
2	6	Presses on the pipette tips while the head is centered above the tip box, even if the tip box is only partially full. *Note:* A collision will result if the head is offset from the tip box location when pressing on pipette tips.
3	2	Ejects the pipette tips into the seating station.
4	2	If applicable, picks up any columns of excess pipette tips from the seating station. The number of excess tips is calculated from the Application Settings. If Application Settings specify that the number of columns of pipette tips in the tip box matches the number of columns of pipette tips to be transfered into the seating station, this step is skipped. *Note:* The head is offset to the right of the seating station, instead of centered above the station, when picking up excess columns of pipette tips.
5	6	If applicable, ejects any excess pipette tips back into the tip box. *Note:* Excess tips are always returned to the left side of the tip box, starting at column 1.
6	2	If applicable, picks up pipette tips from the seating station and moves them to the columns specified in the form. If the pipette tips are already in the correct location after the initial transfer, this step is skipped.
7	1	Moves to a safe height above the wash station.

Reagent Aliquot v2.0 User Guide



The Reagent Aliquot utility aliquots a reagent from a single column or reservoir of a Bulk Reagent storage plate into 1 to 12 columns of a 96-well microplate (Reagent Aliquot plate). The utility uses a single column of eight Agilent 250-µL pipette tips to prepare the aliquots.

This utility is a useful starting point for most applications and workflows, because it simplifies the process of preparing reagent plates for an automation run.

Before you start

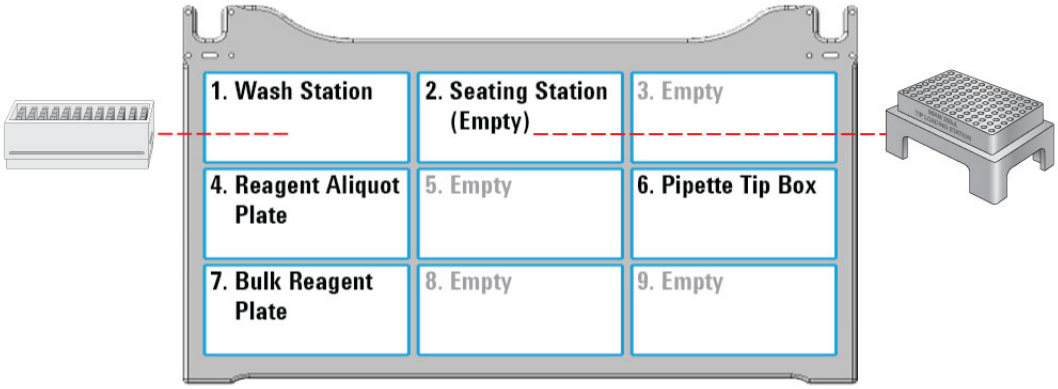
Labware

The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware options	Manufacturer part number*	Deck location
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4, 7
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	4, 7
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4, 7

Labware options	Manufacturer part number*	Deck location
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	4, 7
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	4, 7
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	4, 7
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	4, 7
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	4, 7
96 Nunc 269620, Flat Bottom, Polystyrene	Thermo-Fisher 269620	4, 7
96 Eppendorf 96-500 V-bottom, Clear, PolyPro	Eppendorf 96/500	4, 7
96 Eppendorf 96-1000 U-bottom, Clear, PolyPro	Eppendorf 96/1000	4, 7
96 Waters 186005837, Clear PolyPro	Waters 186005837	4, 7
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	7
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	7
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottom	Agilent 201254-100	7
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	7
96 V11 Manual Fill Reservoir	Agilent G5498B#049	7
*For dimensionally equivalent alternatives, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.		

Reagents

- When preparing a reagent plate for another application run, make sure to use the application-specific Reagent Volume Calculator to:
  - Determine the amount of reagent or sample required in each well.
  - Make appropriate labware selections.
  - Calculate bulk reagent volumes required, including required overage volumes.
- Prepare appropriate volumes of bulk reagents, and manually prepare a Bulk Reagent Plate.
- Prepare the Bulk Reagent Plate with the intended reagents. Pipette an excess volume in the plate of at least:
  - PCR plates: 15–20 µL per well
  - U-bottom, V-bottom, and flat-bottom microplates: 25–30 µL per well
  - Deep-well plates: 35–45 µL per well
  - Reservoir plates: varies depending on type

    For details, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

  Note: These overage volumes are higher than in other applications because this utility uses reverse pipetting.

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Look up the recommended values for each labware type in the Labware Reference Guide.**

Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

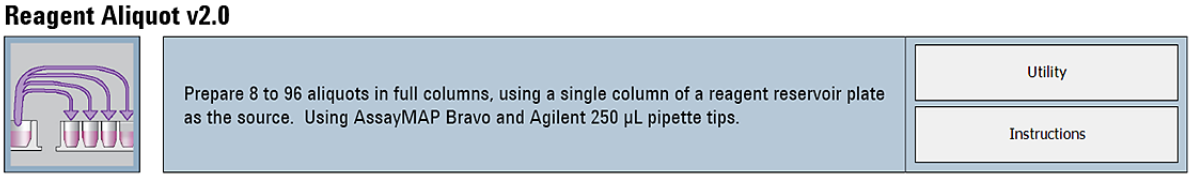
VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Setting up the protocol

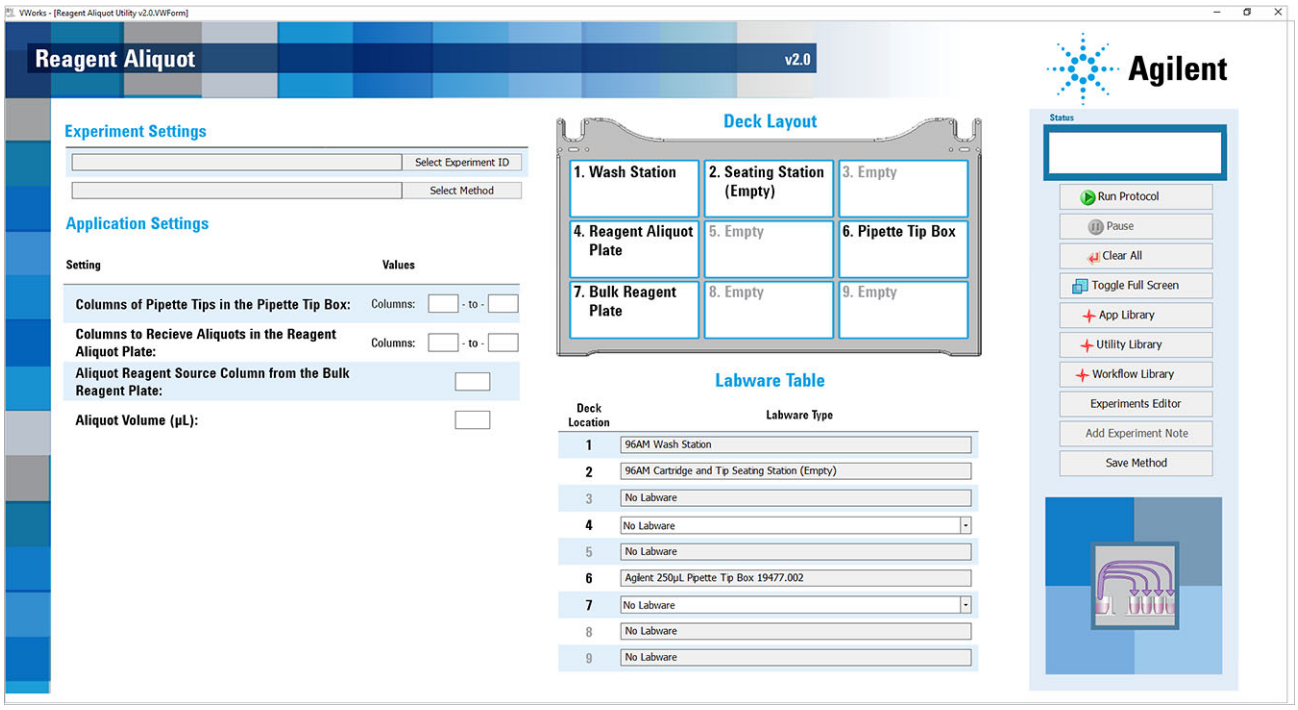
Before starting the protocol, make sure the appropriate selections and values are specified in the Reagent Aliquot utility.

To set up the protocol:

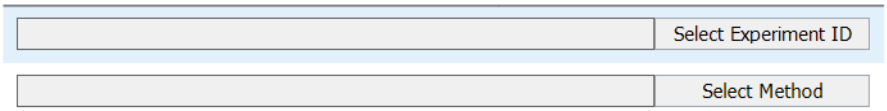
1. Open the **Utility Library**.
2. Locate **Reagent Aliquot**, and then click **Utility**.



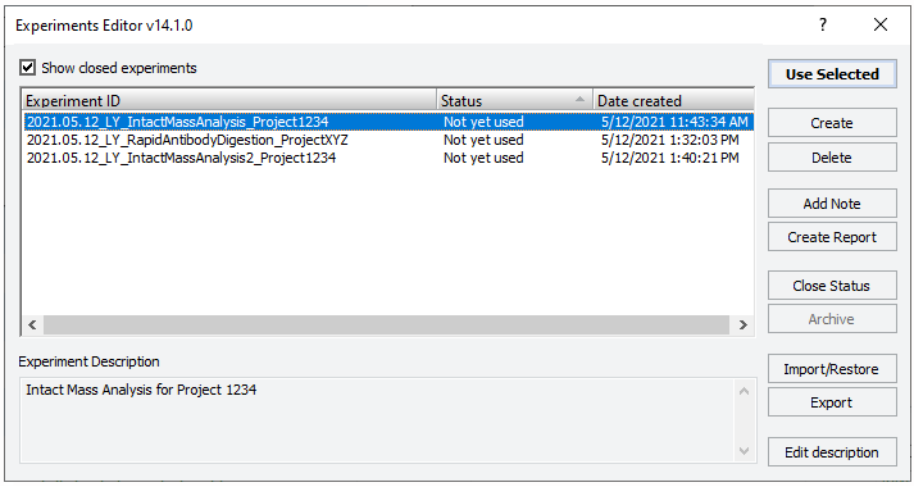
The Reagent Aliquot utility opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

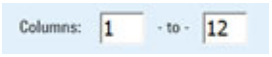
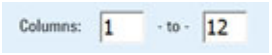
5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Reagent Aliquot run" on page 523.
- To create or modify a method, proceed to step 6.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the following settings for the run:

Setting	Description
Columns of Pipette Tips in the Pipette Tip Box	Specifies the range of pipette tips present in the pipette tip box before the run starts.  • In the left field, specify the column number of the first column of pipette tips present in the tip box. • In the right field, specify the column number of the last column of pipette tips present in the tip box. One full column of pipette tips is used for every run of this utility. The remaining pipette tips are automatically returned to the pipette tip box for use in future runs. **IMPORTANT** The pipette tip box must contain contiguous columns of 8 pipette tips each, within the range of columns specified.
Columns To Receive Aliquots in the Reagent Aliquot Plate	Specifies the range of wells that you want to fill in the Reagent Aliquot Plate.  • In the left field, specify the column number of the first column in the Reagent Aliquot Plate that will be filled with aliquots. • In the right field, specify the column number of the last column in the Reagent Aliquot Plate that will be filled with aliquots.
Aliquot Reagent Source Column from the Bulk Reagent Plate	Defines the column of the Bulk Reagent Plate that contains the bulk reagent to be aliquoted into the Reagent Aliquot Plate.
Aliquot Volume (µL)	Defines the volume of bulk reagent to be aliquoted to each of the selected wells in the Reagent Aliquot Plate. *Note:* The overage volume is not automatically included in this value. If overage is required for your application, ensure that this value is adjusted to include overage.

7 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

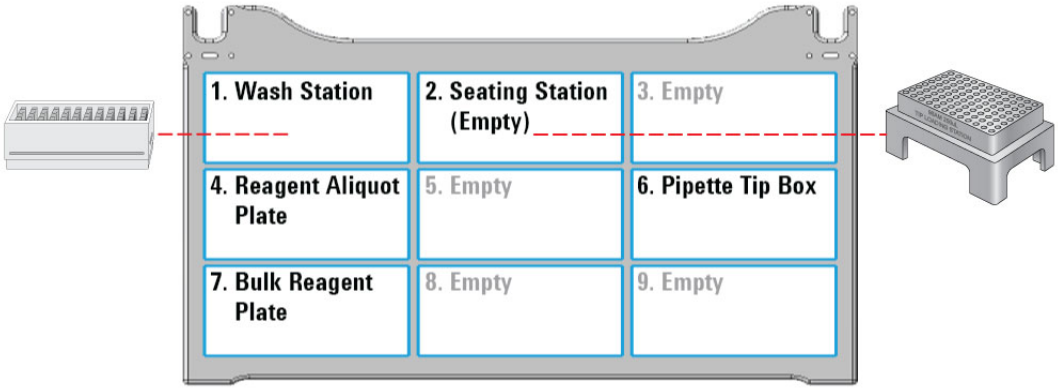
Starting the Reagent Aliquot run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories, filled reagent plates, and collection plates are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

Make sure the labware are properly seated on the Bravo deck.

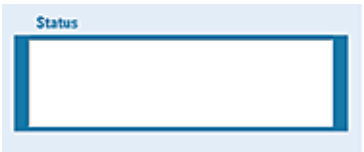


CAUTION **A collision can occur if any pipette tips are present in the columns outside the specified range.**

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



When the run is finished, remove the labware from the deck.

WARNING **To stop a run in an emergency, use the emergency stop pendant.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

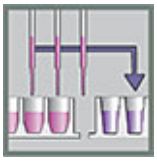
Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Reagent Aliquot protocol using the Default Method settings. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
1	1	Dispenses any liquid remaining in the syringes into the wash station.
2	2	Performs Tips Off to ensure no tips remain on the head at the beginning of the run.
3	1	Performs two syringe drying cycles by aspirating and dispensing air and then conducting at tip touch across the wash station chimneys.
4	6	Presses on all the pipette tips in the tip box. Although all the pipette tips are picked up from the tip box at this step, only 1 column of pipette tips is used for this utility.
5	2	Ejects the pipette tips into the seating station. *Note:* The head is centered over the seating station when ejecting the pipette tips.
6	2	If applicable, picks up any columns of excess pipette tips from the seating station. *Note:* The head is offset to the right side of the seating station when picking up columns of excess pipette tips.
7	6	If applicable, ejects the excess pipette tips back into the tip box. *Note:* Excess pipette tips are always returned to the left side of the tip box, starting at column 1.
8	2	Presses on a single column of pipette tips from the seating station using the left-most column of syringes.
9	7	Aspirates bulk reagent into the pipette tips from the Bulk Reagent Plate. *Note:* This step uses *reverse* pipetting, which involves aspirating a small excess of liquid into the pipette tips to ensure accurate pipetting for the final aliquot of a set.
10	4	Dispenses the reagent into the Reagent Aliquot Plate. The utility uses either of the following two dispense modes: • Multiple low-volume aliquots are dispensed from a single aspirate step. • A single high-volume aliquot requires multiple aspirate steps. In both cases, a small excess volume of the bulk reagent remains in the pipette tips because of the reverse pipetting technique used.

Protocol step	Head moves to deck location...	Action
11	7 & 4	Step 9 and Step 10 are repeated until all aliquots have been prepared in the Reagent Aliquot Plate.
12	7	Dispenses excess reagent within the pipette tips back into the Bulk Reagent Plate.
13	2	Ejects the used pipette tips back into the seating station. *Note:* The used pipette tips are always ejected to column 1 of the seating station.
14	1	Moves the head to a safe distance above the wash station.

Reagent Transfer v3.0 User Guide



The Reagent Transfer v3.0 utility transfers samples or reagents from a Source Plate to a Destination Plate. This utility is useful for a wide variety of sample manipulation and reagent preparation operations. The utility features include the ability to:

- Conduct liquid transfers using either the bare probes of the AssayMAP head or 250-µL pipette tips.
- Control the liquid-handling processes, including:
  - Use reverse pipetting or forward pipetting modes, which can increase pipetting accuracy depending on the liquid properties and volume being transferred.
  - Select from a list of liquid classes or enter a custom liquid class.
  - Specify whether to conduct syringe drying cycles and pre-wet cycles.
- Control the locations of the liquid transfer in both the Source and Destination plates.

Before you start

Labware

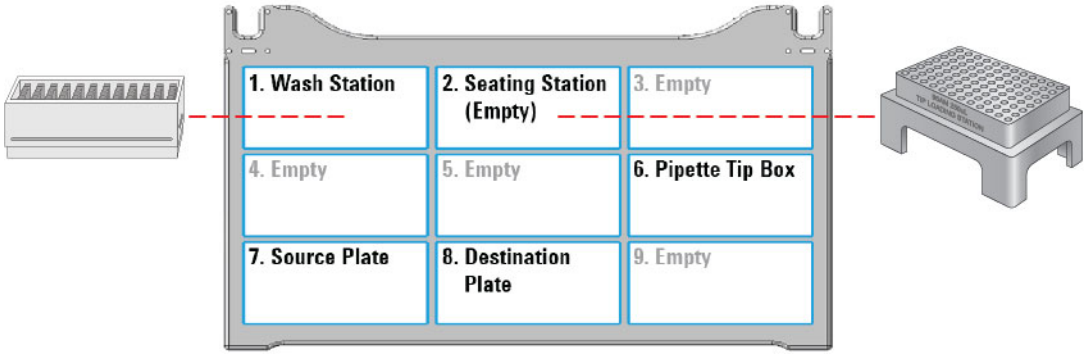
The following table provides a complete list of labware options and the corresponding deck locations for the Source Plate and Destination Plate.

The following figure shows the nine Bravo deck locations for labware.

CAUTION

Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.

Figure Labware locations on the Bravo deck (top view)



Labware options for deck locations 7 and 8	Manufacturer part number*	Source Plate volume overage (well)**
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	10 µL
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	10 µL
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	10 µL
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	30 µL
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	20 µL
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	20 µL
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	20 µL
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	20 µL
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	25 µL
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	3 mL
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	4.5 mL
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottom	Agilent 201254-100	20 mL
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	20 mL
96 V11 Manual Fill Reservoir	Agilent G5498B#049	35 mL
96AM Receiver Plate	Included with Agilent cartridge rack	Not applicable
96 Eppendorf 96-500 V-bottom, Clear, PolyPro***	Eppendorf 96/500	25 µL
96 Eppendorf 96-1000 U-bottom, Clear, PolyPro***	Eppendorf 96/1000	25 µL
96 Waters 186005837, Clear PolyPro***	Waters 186005837	30 µL

Labware options for deck locations 7 and 8	Manufacturer part number*	Source Plate volume overage (well)**

*For dimensionally equivalent alternatives, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

**This overage does not include the volume required for reverse pipetting. The volume is listed by well, where a well is defined as an individual well in a 96-well plate. But in labware where multiple syringes can draw from a common source, a well is defined as that common source. For example, a 12-Column Low-Profile Reservoir has 12 wells, an 8-Row Low-Profile PolyPro Reservoir has 8 wells, and a Seahorse 201254-100, PP, No Walls, Pyramid-bottom Reservoir has only 1 well.

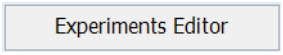
***Pipette tips are required for reagent transfers using these deep-well plate options.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

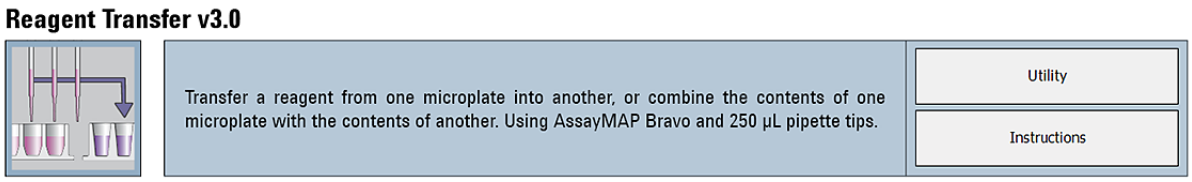
VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Setting up the protocol

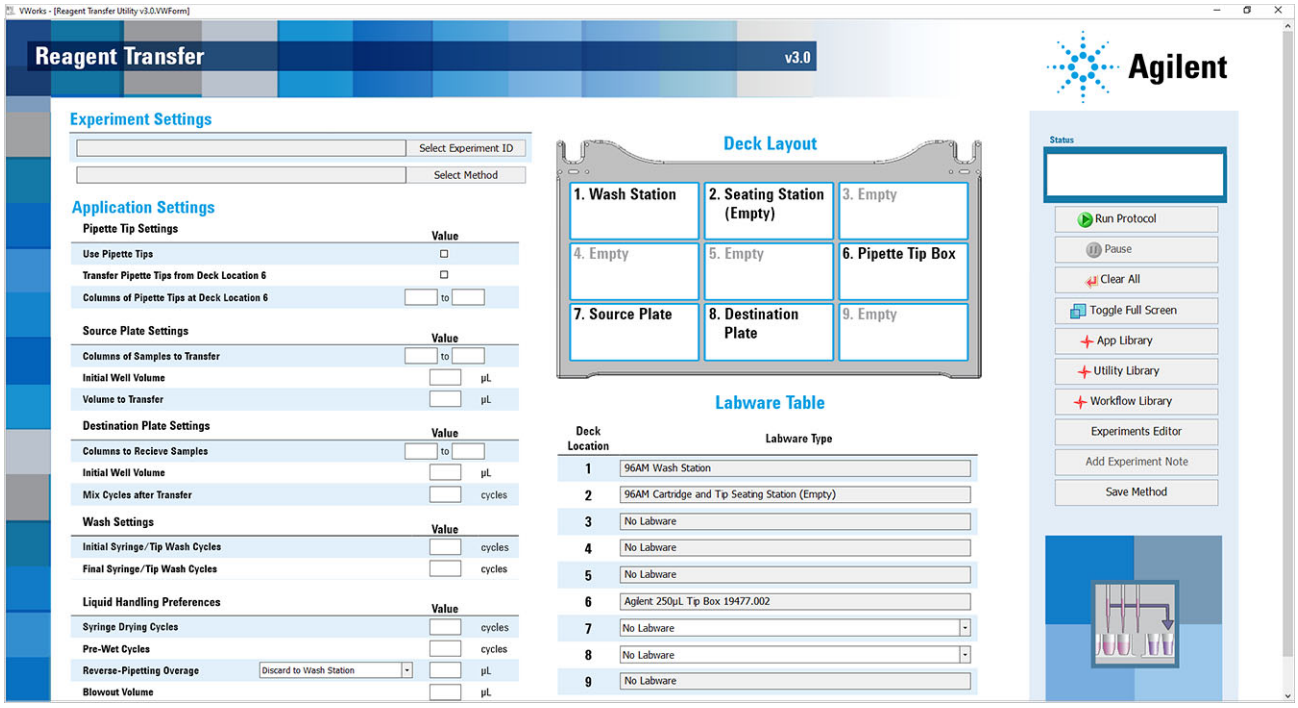
Before starting the protocol, make sure the appropriate selections and values are specified in the Reagent Transfer utility.

To set up the protocol:

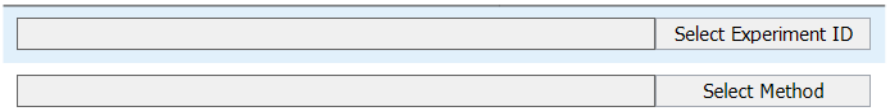
1. Open the **Utility Library**.
2. Locate **Reagent Transfer**, and then click **Utility**.



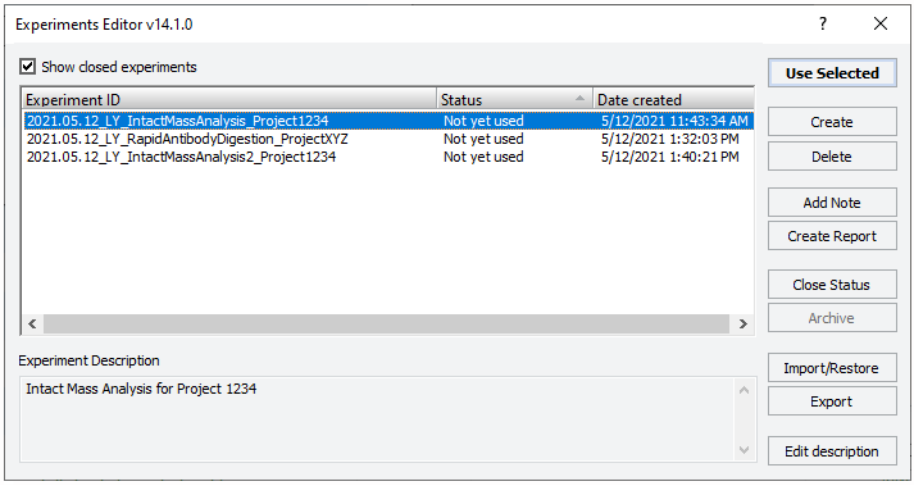
The Reagent Transfer utility opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Reagent Transfer run" on page 536.
- To create or modify a method, proceed to step 6.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the following settings for the run:

a Specify the **Pipette Tip Settings.**

Pipette Tip Setting	Description
Use Pipette Tips	The option to use 250 µL pipette tips to transfer the liquid: • To use pipette tips, select the check box. • To use the bare probes on the AssayMAP head, clear the check box. *Note:* Ensure that the labware selection is appropriate for the bare probes. For details, see Before you start. Default: Not selected

Pipette Tip Setting	Description
Transfer Pipette Tips from Deck Location 6	If the **Use Pipette Tips** check box is selected, specify whether to transfer pipette tips using the Reagent Transfer utility. **CAUTION** **A collision can occur if any pipette tips are present in the source tip box outside of the range specified on the form.** • To transfer the pipette tips from a source tip box at deck location 6 to the **empty** seating station at deck location 2, select the check box. **IMPORTANT** The pipette tip box must contain contiguous columns of 8 pipette tips. • To use pipette tips that are already present in the seating station at deck location 2, clear the check box. For example, if you do a run and transfer the pipette tips from deck location 2 to the seating station, and then want to do additional runs with the same settings, the pipette tips can be reused. **IMPORTANT** The pipette tips must be in contiguous columns of 8 pipette tips. Default: Not selected
Columns of Pipette Tips at Deck Location 6	If the **Transfer Pipette Tips from Deck Location 6** check box is selected, specify the range of full columns of pipette tips present in the source tip box (deck location 6) at the beginning of the run: • Left field is the first column that contains tips. • Right field is the last column that contains tips. For example: • For a full tip box: left field = 1, right field = 12 • For partially filled tip box with tips in columns 6 - 10: left field = 6, right field = 10 Default: 1 to 12 Range: • Starting column: 1–12 • Ending column: 1–12

b Specify the **Source Plate Settings.**

Source Plate Setting	Description
Columns of Samples to Transfer	The range of wells (full columns) in the Source Plate (deck location 7) to be used for the reagent transfer. *Note:* If using bare probes, use the default range of 1 to 12. When using bare probes, the head moves as if it is moving all 96 positions from the Source Plate to the Destination Plate. Therefore, liquid will be moved from every position on the Source Plate where it exists to every corresponding position on the Destination Plate. Default: 1 to 12 Range: • Starting column: 1–12 • Ending column: 1–12
Initial Well Volume	The volume of liquid present in the wells of the Source Plate (deck location 7) before transferring the liquid. For labware where multiple syringes draw from a common source, a "well" is defined as that common source. For example, a 12-Column Low-Profile Reservoir has 12 wells, an 8-Row Low-Profile PolyPro Reservoir has 8 wells, and a Seahorse 201254-100, PP, No Walls, Pyramid-bottom Reservoir has only 1 well. For these labware types, the Initial Well Volume is calculated as: $$\frac{\text{Overage Volume of "Well"} + (\text{Volume to Transfer per Syringe} \times \text{Number of Syringes})}{\text{Number of Syringes Drawing from "Well"}}$$ For example, in the Seahorse 201254-100, PP, No Walls, Pyramid-bottom Reservoir the overage required is 20 mL plus 96 times the volume to be transferred (e.g., 100 µL) divided by 96: $$\frac{20\ \text{mL} + 0.96\ \text{mL} = 20.96\ \text{mL}}{96} = 0.218\ \text{mL}$$ This value is used to calculate the appropriate height for the pipette tips or probes during the aspiration process, and to facilitate proper dynamic-tip behavior. *Note:* Using a value of 0 will disable dynamic-tip movement and force the probes or tips to aspirate from the minimum distance from the well bottom of the selected labware. This might result in the pipette tip or probe exterior being exposed to liquid that might not be removed at the wash station. Default: 0 (µL) Range: 0–1500 (µL)

Source Plate Setting	Description
Volume to Transfer	The volume of liquid per syringe to transfer from the Source Plate (deck location 7) to the Destination Plate (deck location 8). If this volume setting is greater than the capacity of the probes or pipette tips, the transfer will be evenly split into multiple aspirate-and-dispense cycles. Default: 10 (µL) Range: 0–1200 (µL) *Note:* The Automatic Liquid Class setting is designed for a 5 µL minimum. A volume less than 5 µL might require a custom liquid class. *Note:* A value of 0 will drive the probes or tips to the minimum height within the wells and keep them there for the duration of the aspiration step.

c Specify the **Destination Plate Settings.**

Destination Plate Setting	Description
Columns to Receive Samples	The range of full columns in the Destination Plate (deck location 8) to which the samples will be transferred. When using pipette tips, this range can differ from the range specified for the **Columns of Samples to Transfer** value, but it must contain the same number of full columns. If not, the software displays an error message, and the protocol automatically aborts. When using bare probes, use the default range of 1 to 12. The AssayMAP Bravo head moves as if it is transferring liquid from all 96 wells. The wells that contain liquid dictate which wells are transferred. Default: 1 to 12 Range: • Starting column: 1–12 • Ending column: 1–12

Destination Plate Setting	Description
Initial Well Volume	The volume of liquid present in each well of the Destination Plate (deck location 7) before starting the transfer. *Note:* A well is defined as an individual well in a 96-well plate. But in labware where multiple probes can pull from the same continuous liquid source, a well is defined as that continuous liquid source. For example, a 12-Column Low-Profile Reservoir has 12 wells, an 8-Row Low-Profile PolyPro Reservoir has 8 wells, and a Seahorse 201254-100, PP, No Walls, Pyramid-bottom Reservoir has only 1 well. This value is used to calculate the appropriate height for the pipette tips or probes during the transfer dispense process, and to facilitate proper dynamic tip behavior. Default: 0 (µL) Range: 0–1500 (µL)
Mix Cycles after Transfer	The number of aspirate-and-dispense cycles used to mix the contents of the Destination Plate (deck location 8) after the transfer has been completed. The volume used for the mixing process is automatically calculated based on practical low-volume pipetting limits and maximum syringe or probe volume capacities. Default: 0 Range: 0–20 Mixing formula: If (x – 50 µL) > Syringe/Tip Capacity, then Mixing Volume = Syringe/Tip Capacity If 50 µL ≤ x ≤ Syringe/Tip Capacity, then Mixing Volume = 0.75x If x < 50 µL, then Mixing Volume = 0.5x where, x is the final volume in the destination wells, maximum Syringe Capacity = 250µL, maximum Tip Capacity = 140µL *Note:* Syringe and pipette tip capacities vary depending on the Blowout Volume setting.

d Specify the **Wash Settings.**

Wash Setting	Description
Initial Syringe/Tip Wash Cycles	The number of syringe or pipette tip wash cycles to be performed at the wash station (deck location 1) before starting the reagent transfer portions of the run. Default: 0 Range: 0–10

Wash Setting	Description
Final Syringe/Tip Wash Cycles	The number of syringe or pipette tip wash cycles to be performed at the wash station (deck location 1) after completing the reagent transfer portions of the run. Default: 0 Range: 0–10

e Specify the **Liquid Handling Preferences.**

Liquid Handling Setting	Description
Syringe Drying Cycles	The number of syringe purge cycles to be conducted with air before starting the run. This step occurs at different times for runs that use pipette tips instead of probes. • **If using pipette tips**. This step occurs before the Initial Wash cycles. • **If using bare probes**. This step occurs after the Initial Wash Cycles are completed. Default: 2 Range: 0–5
Pre-Wet Cycles	The number of times to wet the probes or pipette tips with the liquid from the Source Plate (deck location 7) before starting the transfer process. *Note:* Prewetting the pipette tips is a common pipetting technique that can greatly increase accuracy in certain situations. Default: 0 Range: 0–5
Reverse-Pipetting Overage	Specifies whether to run in reverse pipetting mode, and if so, the volume to be added to the volume that is being transferred. • **To specify forward pipetting mode**. Use the default setting of 0 µL. • **To specify reverse pipetting mode**. Use a value that is greater than 0 µL, and select the discard location for the extra liquid. This extra liquid remains in the pipette tips or probes for the entire reagent transfer process. Before conducting the mix cycles after the transfer, the excess volume is discarded to the Source Plate (deck location 7) or to Wash Station Waste (deck location 1), depending your selection. *Note:* Reverse pipetting is a common pipetting technique that can greatly increase accuracy in certain situations. Default: 0 (µL) Range: 0–50 (µL)

Liquid Handling Setting	Description
Blowout Volume	The volume of air to dispense out of the syringes or pipette tips after completing the liquid transfer steps. The blowout process is different for the reverse-pipetting and forward-pipetting modes because of the presence of the Reverse-Pipetting Overage volume. • **Forward pipetting mode**. The blowout occurs after the transfer has completed, and then again after mixing, if conducted. • **Reverse pipetting mode**. The blowout occurs only after the mixing step, if conducted, because of the presence of the Reverse-Pipetting Overage volume. Default: 0 (µL) Range: 0–50 (µL)
Liquid Class	The pipetting speed and accuracy. To ensure consistent pipetting, always select a liquid class for liquid-handling tasks. Choose from the following options: • **<Automatic>** (default). Automatically assigns one of the following liquid classes, based on whether pipette tips are used and the volume being transferred. These are good general-purpose liquid classes for most reagents and buffers that are used for the AssayMAP system. – Bare probes: 0–20 µL (AM_ProbesLowVol) > 20 µL (AM_ProbesHighVol) – Pipette tips: 0–20 µL (AM_250uLTipsLowVol) > 20 µL (AM_250uLTipsHighVol) • **Slow Flow (5 µL/sec).** A slower flow rate is better for viscous solutions. • **Fast Flow (100 µL/sec).** A faster flow rate is a good starting point for high organic solutions. If these flow rates do not provide the desired performance, you may enter a custom liquid class. To create a custom liquid class, you use the VWorks Liquid Library Editor. For details on how to open the VWorks Liquid Library Editor, see Creating a custom liquid class. ***To enter a custom liquid class for the reagent transfer:*** In the **Liquid Class** box, type the liquid class name exactly as it appears in the VWorks Liquid Library Editor.

f In the **Labware Table**, select that labware you are using at deck locations 7 and 8.

7 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

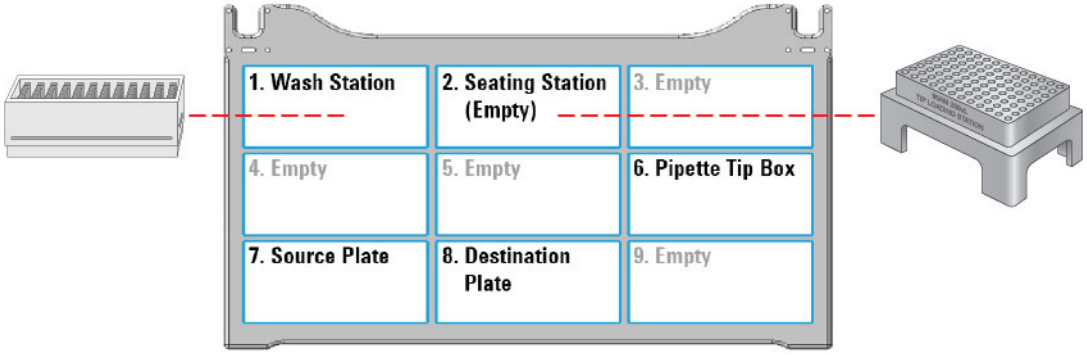
Starting the Reagent Transfer run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

Make sure the labware are properly seated on the Bravo deck.



2 If the method specifies **Use Pipette Tips**, do one of the following:

- If the **Transfer Pipette Tips from Deck Location 6** check box is selected, ensure that the:
  - Pipette tip box containing the specified range of pipette tips is in position at deck location 6.
  - Empty seating station is in place at deck location 2.
- If the **Transfer Pipette Tips from Deck Location 6** check box is cleared, ensure that the seating station at deck location 2 contains the specified range of pipette tips.

  Note: A message will appear and ask you to verify that the pipette tips are in the correct locations. Ensure that the pipette tips are arranged in the specified locations.

CAUTION **A collision can occur if any pipette tips are present in the columns outside the specified range.**

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

3 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

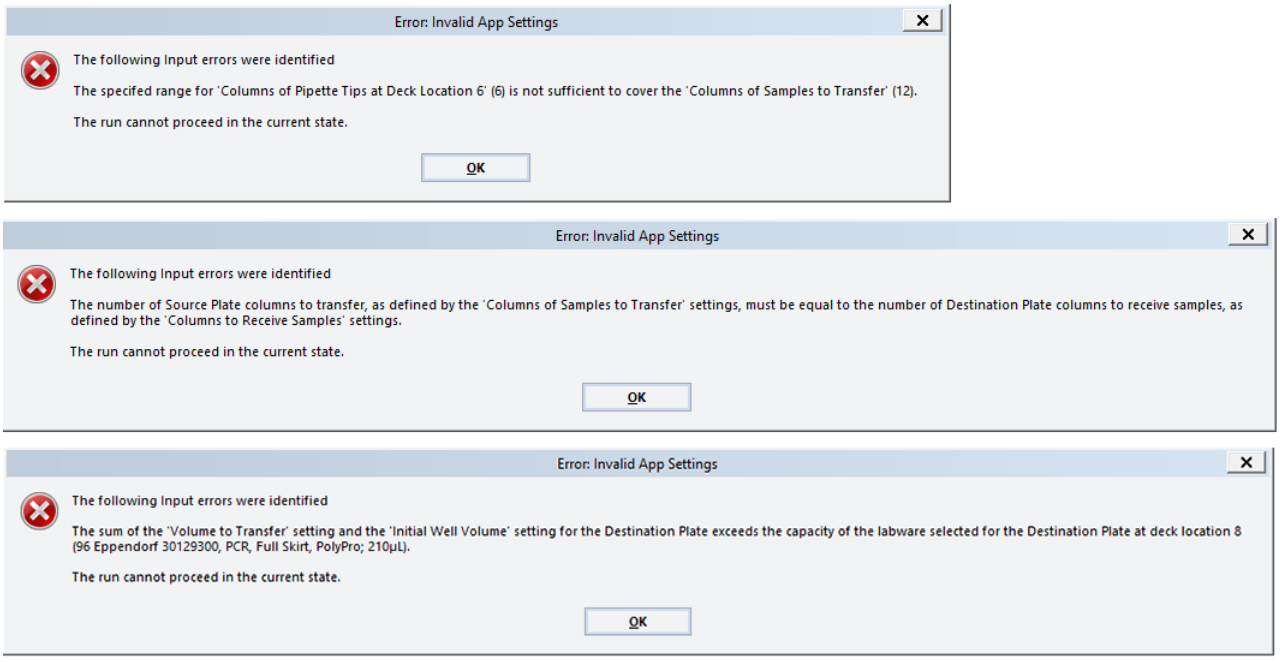
See the following section for guidance on how to handle error, warning, and verification messages. To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Error, warning, and verification messages

Detected error in the setup

If the software detects a conflict in the setup, an error message or warning will appear before the run begins.

Figure Examples of error messages that prevent run execution



To resolve error messages about setup conflicts:

1 In the form, resolve the settings described in the error message, for example:

- Insufficient number of columns of pipette tips

- Unequal number of columns selected for Source and Destination plates
- Incompatible labware selections (labware that require pipette tips)

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

2 To save the new method, click Save Method.

In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

3 To select the new method, click **Select Method.**

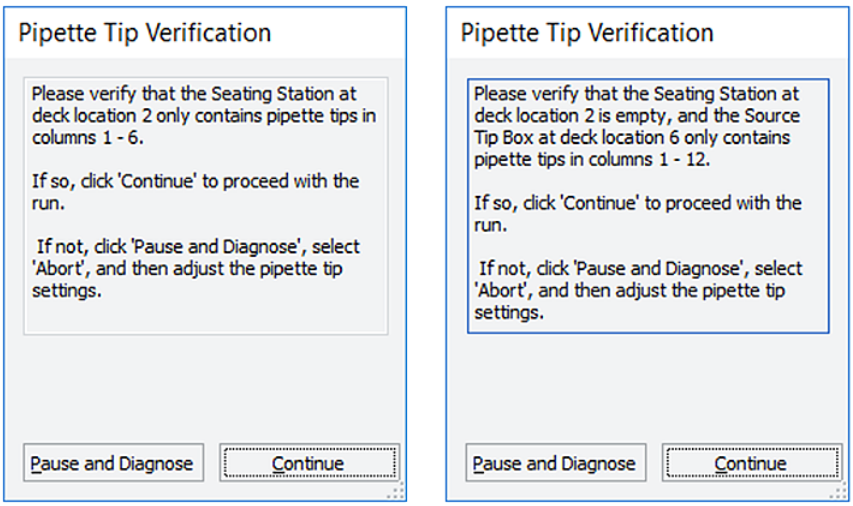
In the **Open File** dialog box, select the new method, and click **Open**.

4 Click Run Protocol to start the run.

Verification and warning messages

Verification and warning messages that appear allow you to continue with the run as specified, while assuming the risk posed by the warning. The following figure shows some examples.

Figure Examples of verification messages



To resolve verification and warning messages:

Follow the on-screen instructions to verify that the settings are correct, and then do one of the following:

- If the settings are correct, click **Continue** to start the run.

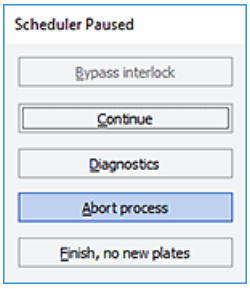
  Note: A Pipette Tip Verification message appears whenever pipette tips are used.

- If you need to change a setting:

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

  a Click **Pause and Diagnose**. The Scheduler Paused dialog box opens.

  b In the **Scheduler Paused** dialog box, click **Abort process**.



c In the form, change the settings to resolve the conflict.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

d To save the new method, click Save Method.

In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

e To select the new method, click **Select Method.**

In the **Open File** dialog box, select the new method, and click **Open**.

f Click Run Protocol to start the run.

Creating a custom liquid class

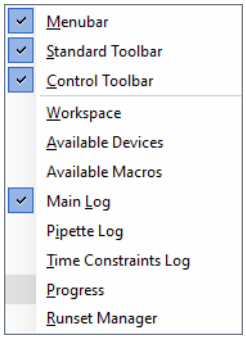
Administrator or technician privileges are required to create or modify liquid classes.

To create a custom liquid class:

1 In the **Reagent Transfer v3.0** form, click **Toggle Full Screen** to change the display of the form so that the VWorks menubar and toolbar are visible, as the following figure shows.

2 In the VWorks window, click **Tools > Liquid Library Editor**. The Liquid Library Editor opens.

If the menubar is not visible in the VWorks window, right-click the window and select **Menubar** in the shortcut menu that appears.



3 For instructions on how to use the Liquid Library Editor, see the section on specifying pipette speed and accuracy in the *VWorks Version 4 Automation Control Setup Guide* in the VWorks Knowledge Base.

Figure VWorks Tools menu and form displayed with full screen off

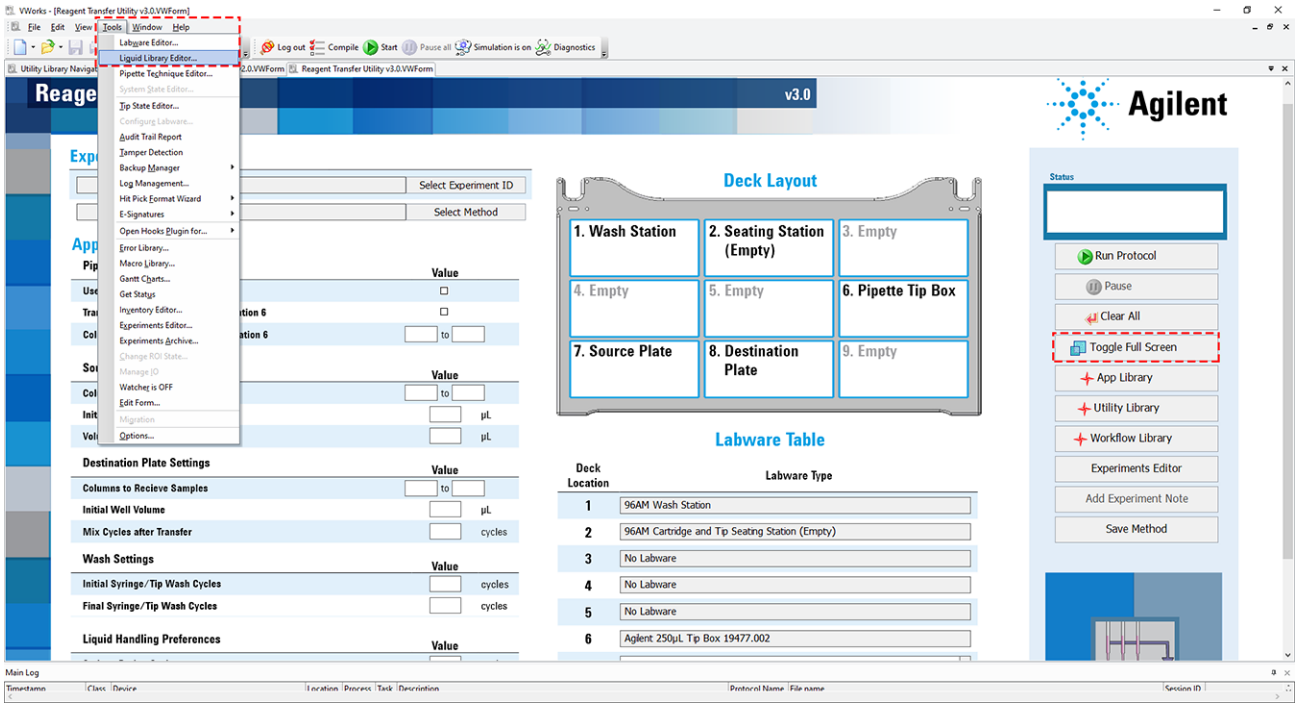
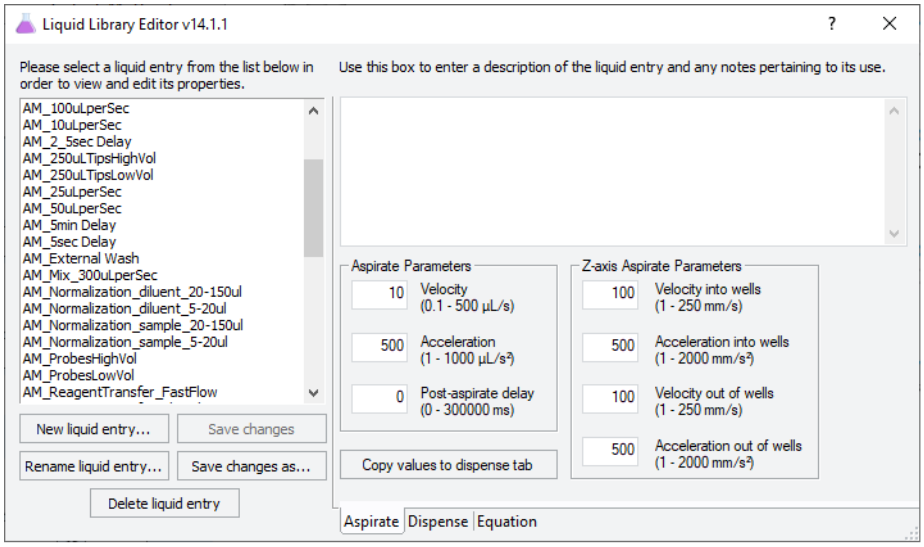


Figure VWorks Liquid Library Editor window



Automation movements during the protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Reagent Transfer protocol using the default method settings. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to deck location...	Action
1	1	Washes the syringes. If pipette tips are used for the transfer, this step occurs after step 3 below to facilitate pipette tip washing.
2	1	Purges the syringe contents to waste for the number of cycles set in the Syringe Drying Cycles setting.
3	6	If applicable, picks up the pipette tips from the source tip box. This step occurs only if the Use Pipette Tips check box and the Transfer Pipette Tips from Deck Location 6 check box are selected.
	2	If applicable, ejects the pipette tips into the seating station.
4	2	If applicable, picks up all the pipette tips from the seating station This step occurs only if the Use Pipette Tips check box is selected and the Transfer Pipette Tips from Deck Location 6 check box is cleared. A confirmation message verifies that the pipette tip locations are correct.
5	7	Aspirates and dispenses the liquid in the Source Plate to wet the pipette tips or syringe probes for the number of cycles set in the Pre-Wet Cycles setting.
6	7	Aspirates the volume to be transferred from the Source Plate using the pipette tips or syringe probes.
7	8	Dispenses the liquid into the Destination Plate.
8	7 & 8	Repeats steps 6 and 7 until the full volume specified in the Volume to Transfer setting has been transferred to the Destination Plate. Pipette tip maximum volume: ~140 µL can be pulled into a mounted tip before the excess fluid overflows into the syringe, because the probe protrudes into the tip volume capacity. Probe maximum volume: 250 µL can be aspirated into the syringe before the syringe is full. The probe maximum volume will be reduced automatically by an amount that is equal to the specified Blowout Volume.
9	8	Mixes the contents of the Destination Plate.
10	8	Performs a blowout slightly above the liquid level of the Destination Plate to clear any remaining liquid.
11	8	Performs a tip touch on the left and right sides of the wells in the Destination Plate.
12	1	Washes the pipette tips or syringe probes.
13	2	If pipette tips are used, ejects the pipette tips into the seating station
14	1	Moves the head above the wash station.

Single Liquid Addition v2.0 User Guide

The Single Liquid Addition Utility is specifically designed to adjust the pH of samples after the samples have been digested using the In-Solution Digestion: Multi-Plate application. This utility enables you to rapidly adjust buffer composition or add a component to up to four sample plates by transferring liquid from a common reagent plate, to up to four independent sample plates. An optional stringent syringe wash step is included to prevent carry over.

Before you start

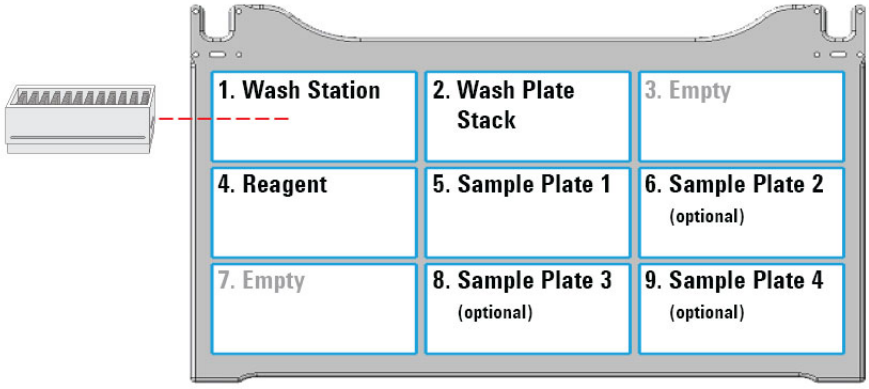
Labware

The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	4
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	4
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	4
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	4

Labware	Manufacturer part number*	Deck location options
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	4
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	4
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	2, 4, 5, 6, 8, 9
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	4
96 V11 Manual Fill Reservoir	Agilent G5498B#049	4

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Reagents

Make sure you:

- Prepare a reagent plate that contains the solution that you want to add to the Sample plates. Place the reagent plate at deck location 4.
- Prepare the Syringe Wash Buffer. For suggested solutions to use, see Preparing the solutions in the "In-Solution Digestion: Multi-Plate v2.0 User Guide" on page 235.
- Ensure that you have four Greiner 650207 White U-Bottom plates for the wash plates. You must fill the plates with the Syringe Wash Buffer, and then run the Plate Stacking protocol to stack the wash plates at Bravo deck location 2. For details, see "Running the Plate Stacking protocol" on page 544.

CAUTION **A small reagent volume excess is required in all labware types to ensure proper volume transfer. Use the Reagent Volume Calculator to automatically include excess volume, or look up the recommended values for each labware type in the Labware Reference Guide.**

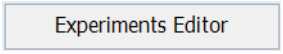
Note: You can find the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

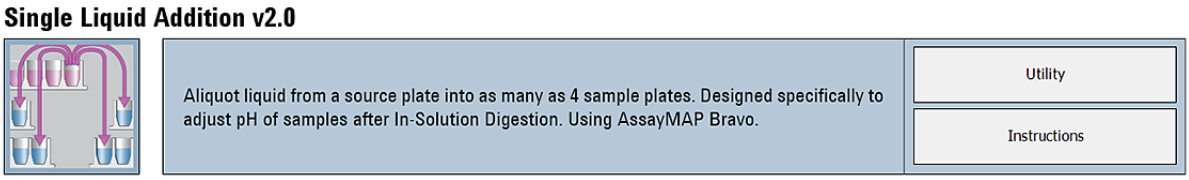
Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

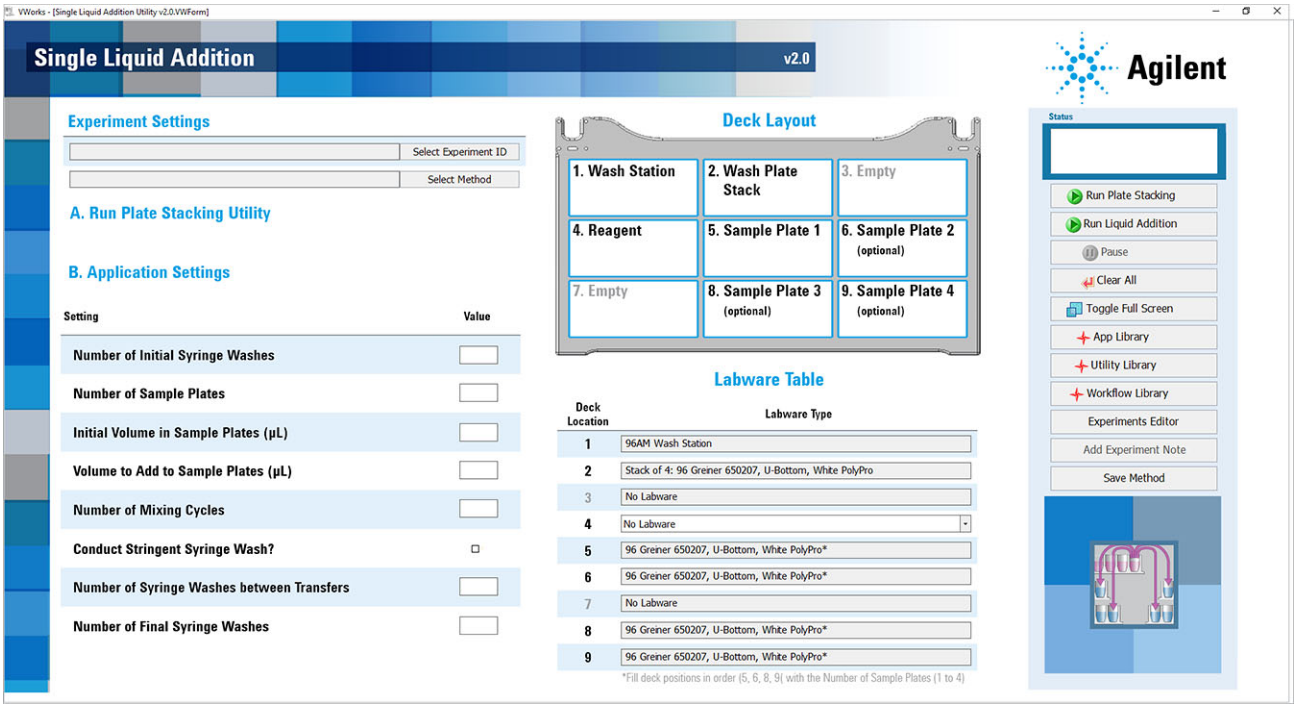
Opening the Single Liquid Addition utility

To open the utility:

1. Open the **Utility Library**.
2. Locate **Single Liquid Addition**, and then click **Utility**.



The Single Liquid Addition utility opens.



Running the Plate Stacking protocol

The Plate Stacking protocol stacks the wash plates properly at Bravo deck location 2 to prevent collision during the Single Liquid Addition protocol run.

IMPORTANT The Single Liquid Addition protocol requires the stack of four wash plates, even if you have less than four sample plates. Ensure that the wash plates are Greiner 650207 White U-Bottom.

Before running the Plate Stacking protocol, prepare four Greiner 650207 White U-Bottom plates as follows:

- Fill the plates with 300 µL Syringe Wash Buffer. The plates will serve as wash plates that are used for cleaning the syringes between liquid transfers.
- Make sure the filled wells correspond to the location of the samples in the sample plates.

To stack the prepared wash plates:

1. In the **Single Liquid Addition** form, click 

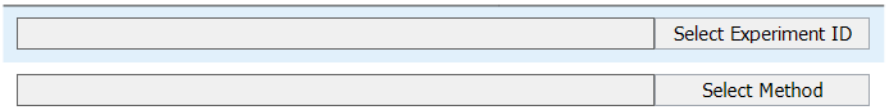
. The protocol starts.
2. Follow the on-screen instructions for placing the wash plates at the appropriate locations on the AssayMAP Bravo deck.
3. Check the **Status** box in the upper right corner of the form. When the Protocol Complete message is displayed, click **OK**.

Setting up the Single Liquid Addition protocol

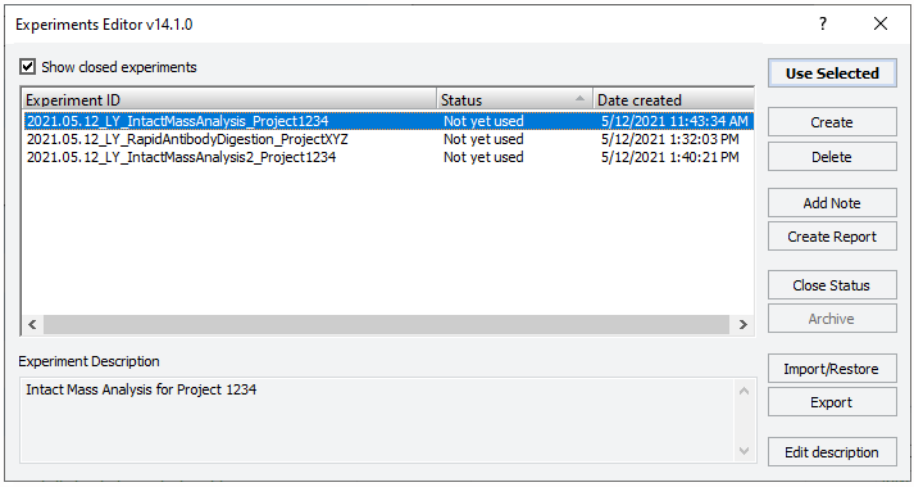
Before starting the protocol, make sure the appropriate selections and values are specified in the Single Liquid Addition utility.

To set up the Liquid Addition protocol:

1. If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



2 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

3 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Single Liquid Addition run" on page 547.
- To create or modify a method, proceed to step 4.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

4 In the **Application Settings** area, specify the following properties for the run:

Property	Description
Number of Initial Syringe Washes	The number of cycles for rinsing syringes with deionized water at the beginning of the run. Range: 0–10 Default: 3
Number of Sample Plates	The number of sample plates (1-4) to which a liquid will be added in this utility. Place only the number of sample plates used on the deck. Positions 2-4 are optional and need not be filled. Range: 1–4 Default: 1
Initial Volume in Sample Plate	The initial volume of the samples. All plates must have the same sample volume. Range: 0–250 Default: 10
Volume to Add to Sample Plate	The volume of solution being added to the samples. This may be limited by the labware. Range: 0–250 Default: 50

Property	Description
Number of Mixing Cycles	The number of mixing cycles that will be used to homogenize the sample liquid and the liquid that is added. Range: 0–20 Default: 10
Conduct Stringent Syringe Wash	The option to wash the syringe with a solution in the wash plate between transfers. Select the check box to perform the wash. Clear the check box to skip the wash. Default: ☐
Number of Syringe Washes between Transfers	The number of Syringe Washes to be performed at the Wash Station, before adding liquid to the next sample plate. Range: 0–10 Default: 5
Number of Final Syringe Washes	The number of times to rinse syringes with deionized water. Range: 0–10 Default: 3

5 In the **Labware Table**, select that labware you are using at deck location 4.

6 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

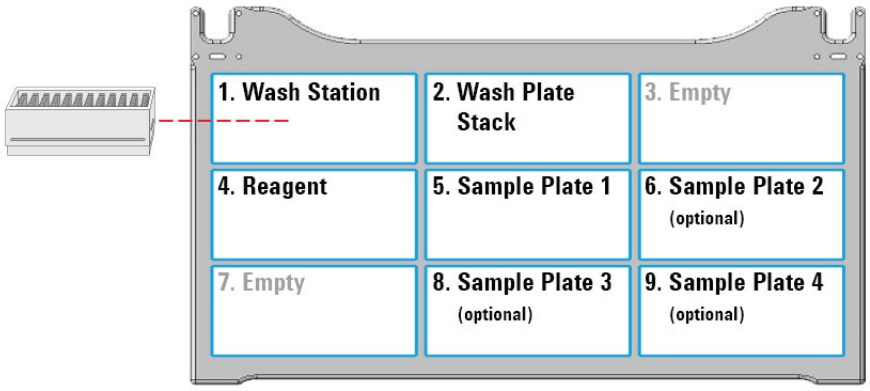
Starting the Single Liquid Addition run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Liquid Addition to start the protocol run.

To monitor the progress of the run, check the **Status** box.

When the run is finished, remove the labware from the deck.

WARNING **To stop a run in an emergency, use the emergency stop pendant.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Syringe Test v2.0 User Guide

The Syringe Test v2.0 utility and the AssayMAP Syringe Test Kit enable verification of the integrity of the probes and syringes in the Bravo 96AM Head.

When to use the AssayMAP Syringe Test Kit

- As a part of routine hardware quality control to verify head functionality
- To verify the functioning of one or more syringes that produce suspicious results
- After a Bravo 96AM Head collision in which syringes may have been damaged
- After replacing any syringes in the Bravo 96AM Head

How it works

The AssayMAP Syringe Test kit contains a rack of resin-free cartridges, labware, and a viscous dye solution. You place the resin-free cartridges and labware containing the dye solution on the Bravo deck at the specified locations, and then run the Syringe Test utility. The Syringe Test protocol does the following:

- Mounts the 96 resin-free cartridges on the syringes in the AssayMAP head.
- Places the cartridge tips in the syringe test solution. The syringes are quickly drawn to a 225 µL draw position. The speed of the aspiration is faster than the liquid can be drawn through the resin-free cartridges into the syringes, so a partial vacuum is formed in the syringes. The cartridges remain in the syringe test solution for 5 minutes to allow the test solution to be drawn into the syringes.
- Ejects the cartridges, and then dispenses the dye solution into a 96-well flat bottom plate.

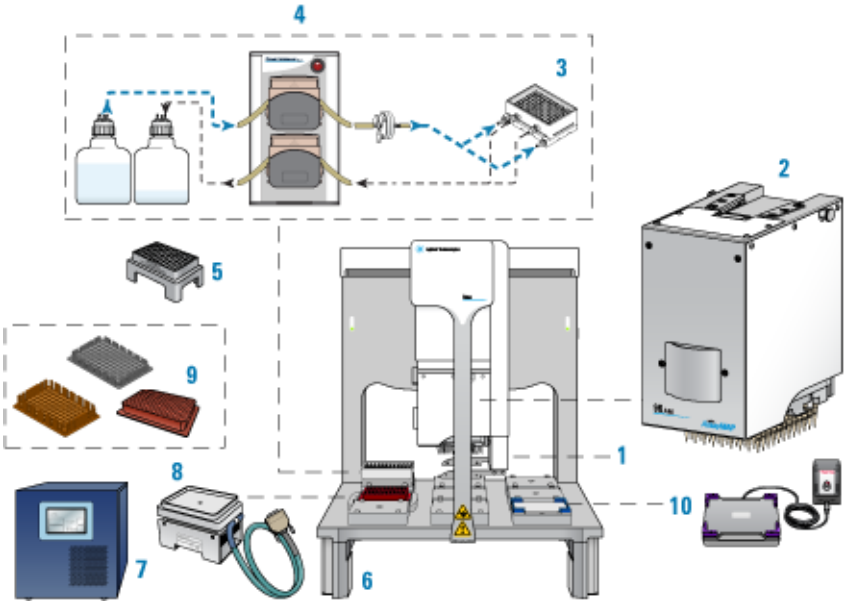
The absorbance of dye solution in each well of the plate is measured using a microplate reader.

If a syringe is damaged or the seal between the syringe probes and the cartridges is compromised, air will be drawn into the syringes from the vacuum. The air leak will decrease the amount test solution drawn into the syringes and have a lower signal identifying the syringe as damaged.

Hardware

The following figure and table show the components of the AssayMAP Bravo Platform, which are required for running the AssayMAP protocols.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station or the later model 96 Channel Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Risers, 146 mm
7	STC controller
8	Peltier Thermal Station with custom plate nest
9	Thermal plate insert
10	Orbital Shaking Station with Control Unit

CAUTION **To avoid a hardware crash and equipment damage, ensure that the wash station contains the white wide-bore chimneys when using the 25 µL cartridges.**

Note: The white wide-bore chimneys work for both 5-µL and 25-µL cartridges and are standard on wash stations purchased in 2020 onward. The wide-bore chimneys are white plastic, whereas the standard-bore chimneys are a semi-clear plastic. For details, see the *96 Channel Wash Station Maintenance Guide*.

Additional required equipment

Required equipment	Description
Centrifuge	Centrifuge with a microplate rotor capable of achieving 500 x g
Microplate reader	Spectrophotometer capable of detecting absorbance at 425 nm in a 96-well microplate and generating a text (TXT) file of the data. *Note:* Although 425 nm is optimal, 405 nm is also acceptable.

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

AssayMAP Syringe Test Kit components

One AssayMAP Syringe Test Kit provides enough materials and reagents to perform 20 tests of the 96 syringes in the Bravo 96AM head.

The following table provides a description of the kit components.

Component	Description	Part Number	Quantity
Syringe Test Kit includes		G5496-60050	1
• AssayMAP Resin-Free Cartridges	96 cartridges used for testing	G5496-60009	1 rack
• Test solution	Aqueous colormetric solution with appropriate viscosity	5190-7471	3 bottle (250 mL each)
• Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottom	Required reusable labware	201254-100	2

Component	Description	Part Number	Quantity
• 96 Greiner 655101 PS Clr Rnd Well Flat Btm	Required reusable labware	655101	2

Labware requirements

The Syringe Test requires the following labware:

- Open reservoir, (2 per test) to hold water (deck location 4) and diluted test solution (deck location 7).
- 96-well microplate, (2 per test) for test sample collection and a centrifugation balance plate (deck locations 8 and 9).

The following figure shows the labware locations on the Bravo deck. The table lists the options for labware type by deck location.

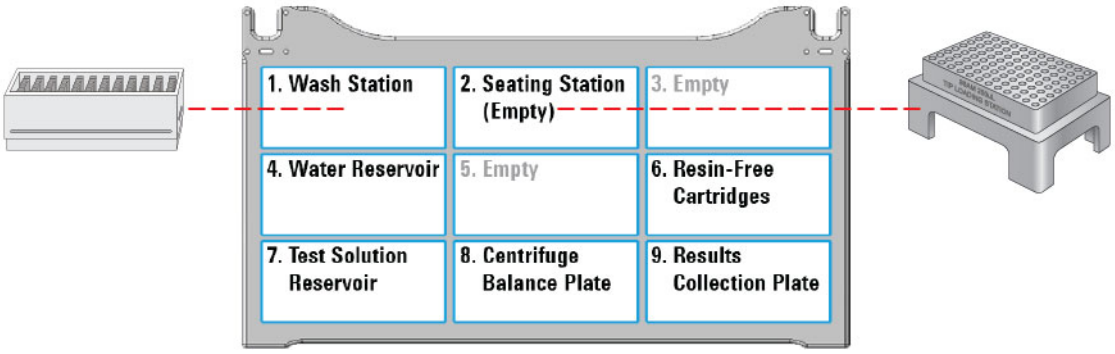


Table Labware options by deck location

Labware options	Manufacturer part number	Deck location
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottom	Agilent 201254-100	4, 7 (default)
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	4, 7
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	4, 7
96 V11 Manual Fill Reservoir	Agilent G5498B#049	4, 7
96 Greiner 655101 PS Clr Rnd Well Flat Btm	Greiner Bio-One 655101	8, 9 (default)
96 Nunc 269620, Flat Bottom, Polystyrene	Thermo-Fisher 269620	8, 9
96 Corning 9017, Flat bottom, polystyrene, clear	Corning 9017	8, 9

Workflow

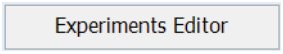
Step	For this task...	See...
1	Run the Startup protocol in the System Startup/Shutdown utility.	"Setting up the Startup protocol" on page 576
2	Set up the Syringe Test protocol.	"Setting up the Syringe Test protocol" on page 553
3	Set up the AssayMAP Bravo deck for the syringe test.	"Setting up the Syringe Test protocol" on page 553
4	Run the Syringe Test.	"Starting the Syringe Test protocol run" on page 555
5	Analyze the results.	"Analyzing the results" on page 559
6	Verify defective syringes.	"Verifying defective syringes" on page 565

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

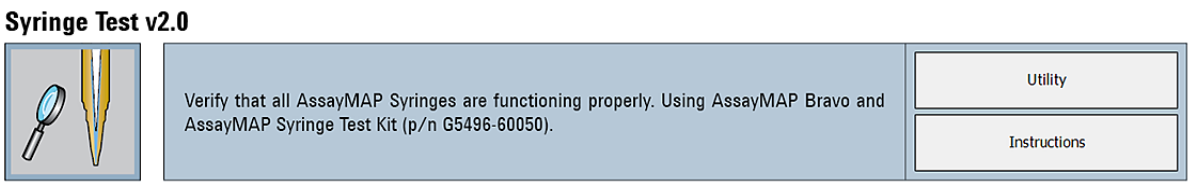
Setting up the Syringe Test protocol

The following procedure specifies volume requirements for the default labware selections. If you choose different labware types from the list of options, you should adjust the volumes accordingly.

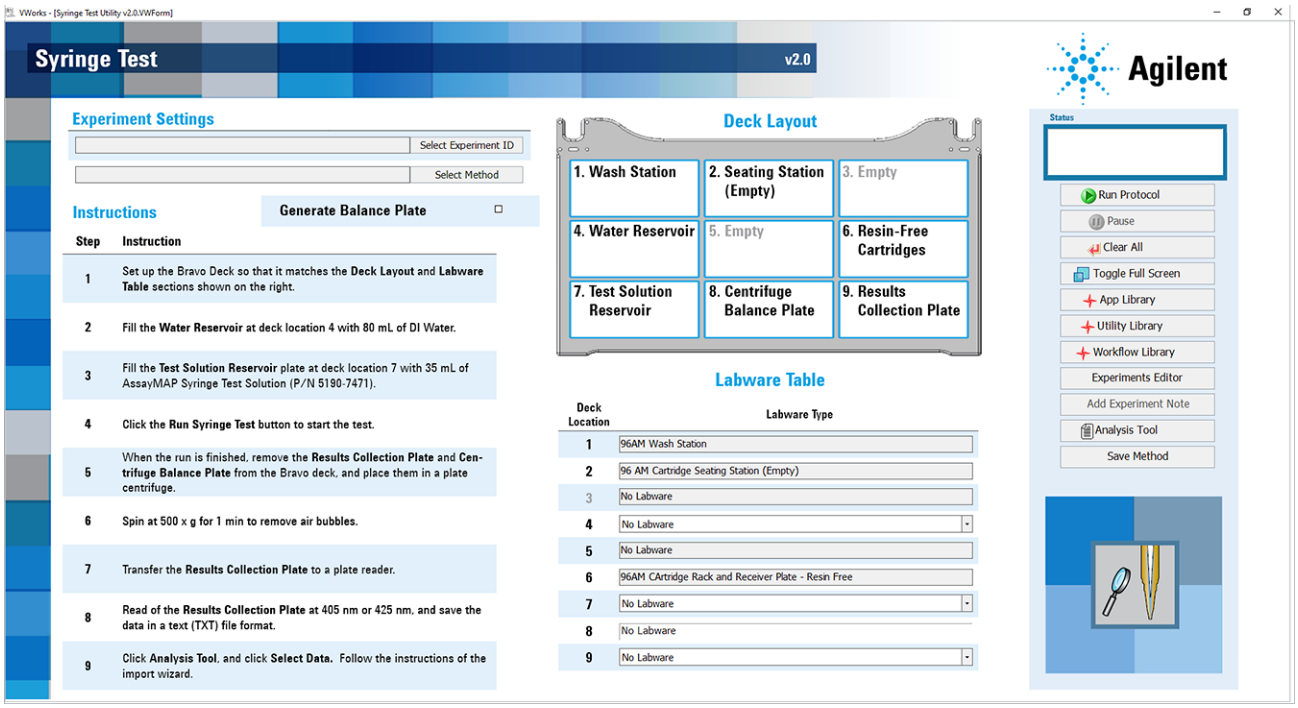
To set up the protocol:

1 Open the **Utility Library**.

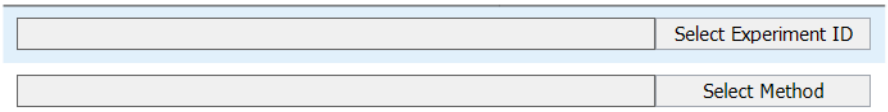
Locate **Syringe Test**, and then click **Utility**.



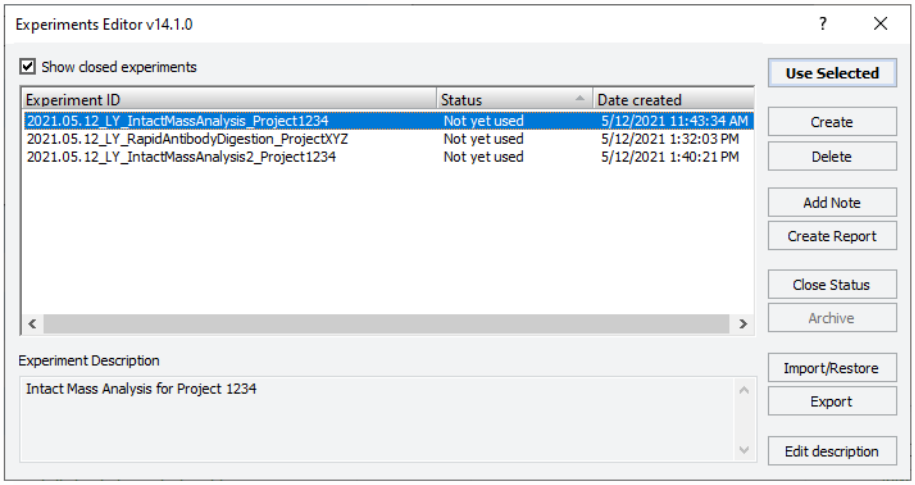
The Syringe Test utility opens.



2 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



3 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

4 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Syringe Test protocol run" on page 555.
- To create or modify a method, proceed to step 5.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

5 In the **Labware Table** area, select the labware you are using for the protocol run.

6 *Optional*. To have the Centrifuge Balance Plate filled with 200 µL water by the Bravo Platform, select the **Generate Balance Plate** check box.

7 To save the method:

a Click Save Method.

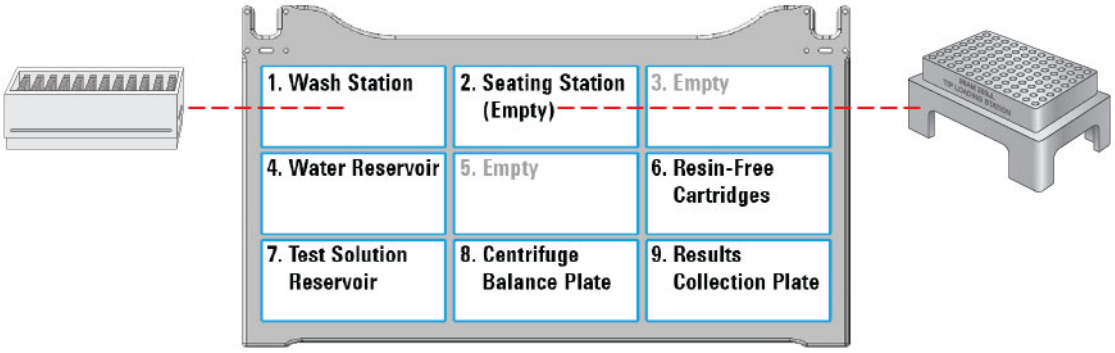
b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

Starting the Syringe Test protocol run

To start the Syringe Test protocol run:

1 Place the accessories or components on the AssayMAP Bravo deck.



Deck location	Accessory or component	Purpose
1	96AM Wash Station or the later model 96 Channel Wash Station	Washes syringes and exterior cartridges
2	96AM Cartridge & Tip Seating Station	Ensures proper seating of the cartridges on the head
4	Reservoir, Seahorse 201254-100, PP (default)*	Holds water for the balance plate and dilution of test solution.
6	Cartridge rack with 96 resin-free cartridges	Holds test cartridges at beginning and end of protocol
7	Reservoir, Seahorse 201254-100, PP (default)*	Holds the 1X test solution
8	96 Greiner 655101 PS Clr Rnd Well Flat Btm (default)*	Microplate of same type as the collection plate for balancing in the centrifuge
9	96 Greiner 655101 PS Clr Rnd Well Flat Btm (default)*	Receives the test solution from the syringes and is used for the absorbance read.

* For other options, see "Labware requirements" on page 552.

2 Ensure the following:

- The physical layout on the Bravo deck matches the **Deck Layout** image in the form.
- The selections in the **Labware Table** reflect the labware you are using for the protocol run.
- The labware are properly seated on the Bravo deck.

CAUTION

Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.

3 Dispense 80 mL of water into the reservoir at deck location 4.

4 Dispense 35 mL of the Test Solution into the reservoir at deck location 7.

5 Click Run Protocol to start the run.

After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol.

WARNING **To stop a run in an emergency, use the emergency stop pendant.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

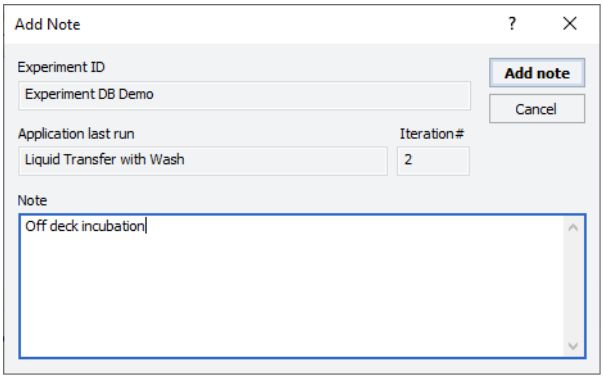
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Automation movements during the Syringe Test run

This section describes the basic movements of the AssayMAP Bravo Platform during the Syringe Test protocol using the Default Method settings. Changing the selections or parameters will alter the movements.

Protocol step	Head moves to location...	Action
Start protocol	1	Dispenses any liquid remaining in the syringes into the wash station between the chimneys.
	1	Washes the syringes.
Cartridge transfer	6	Mounts the resin-free cartridges on the head.
	2	Parks the cartridges in the seating station.
Water transfer	4	Aspirates 125 µL of water.
	9	Dispenses 100 µL water.
	1	Dispenses 25 µL water.
	4	Mixes 5 times, and then aspirates 200 µL of water.
	8	Dispenses 200 µL of water.
Internal Cartridge Wash	4	Aspirates 150 µL water.
	2	Dispenses 10 µL of water into the cartridge cups.
	2	Mounts the cartridges on the head.
	1	Dispenses 100 µL of water through the cartridges into the wash station between the chimneys.
	1	Washes the exterior tips of the cartridges.
	2	Parks the cartridges in the seating station.

Protocol step	Head moves to location...	Action
Test Solution transfer	1	Dispenses 40 µL of water into the wash station between the chimneys.
	2	Aspirates a 25 µL air gap.
	2	Mounts the cartridges on the head.
	7	Submerges the cartridges in Test Solution. Rapidly aspirates 225 µL Test Solution and equilibrates for 5 min with the cartridge tips submerged.
	2	Parks the cartridges in the seating station.
	1	Dispenses 125 µL of Test Solution into the wash station between the chimneys, and then washes the exterior of the syringe probes.
	9	Dispenses 100 µL of Test Solution and mixes it.
	9	Dispenses 5 µL of the air gap.
	1	Dispenses remaining 20 µL of the air gap.
	1	Washes the syringes.
Cup wash	4	Aspirates 50 µL of water.
	2	Washes the cartridge cups and exercises the cartridges off task.
	1	Dispenses the water into the wash station between the chimneys, and then does an external probe wash.
Internal Cartridge Wash	4	Aspirates 150 µL water.
	2	Mounts the cartridges on the head.
	1	Dispenses the water into the wash station between the chimneys, and then washes the exterior of the cartridge tips.
	6	Ejects the cartridges.
Final syringe wash	1	Washes the syringes.

Analyzing the results

How the data is analyzed

The absorbance measurement of each well is compared to an expected reference value, which is determined from a statistical analysis of the data from all wells.

Any syringe that has a corresponding value $\geq$ 10% of the reference value fails the test.

To analyze the syringe test results:

1. After the run is completed, remove the collection microplate and the balance microplate from the Bravo deck and spin them in a centrifuge at 500 x g for 1 min to remove any bubbles within the wells.
2. Set up the plate reader to:

- Detect absorbance at 425 nm

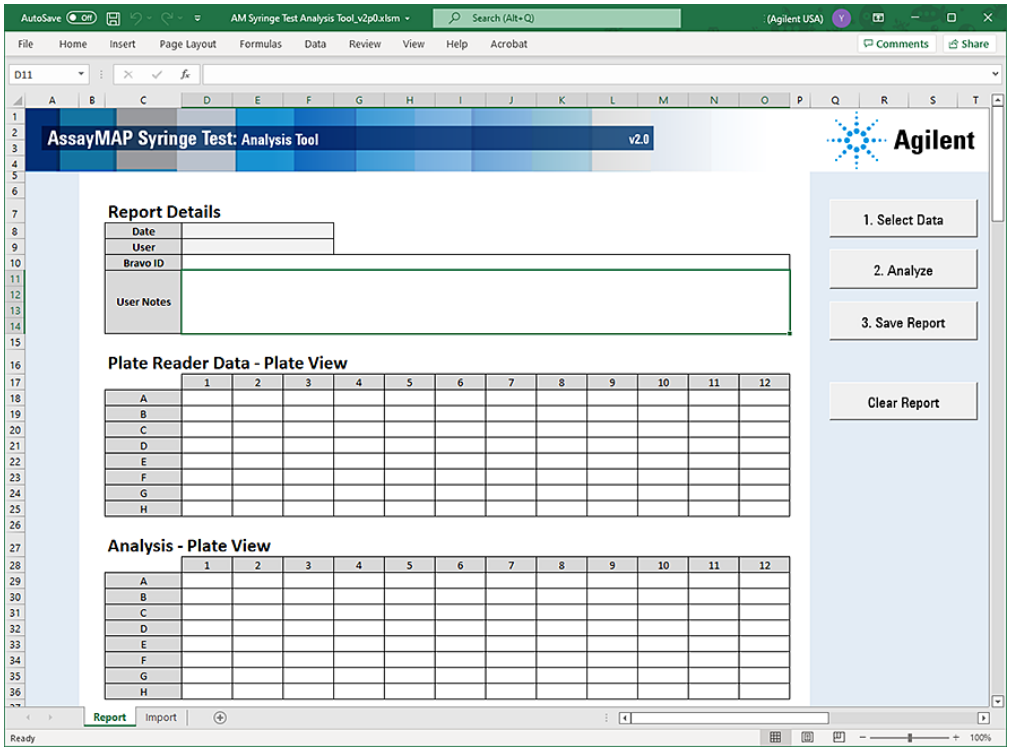
  Note: Although 425 nm is optimal, 405 nm is also acceptable.
- Save the results to a file with a text (TXT) file format

  Note: Alternatively, you can save the data to a Microsoft Excel file (XLSX) and then use the copy and paste commands to transfer the data to the Analysis Tool.

3 Transfer the file to the computer running the Protein Sample Prep Workbench.

4 In the **Syringe Test** form, click Analysis Tool to open the AssayMAP Syringe Test Analysis Tool.

The AssayMAP Syringe Test Analysis Tool opens in Microsoft Excel.



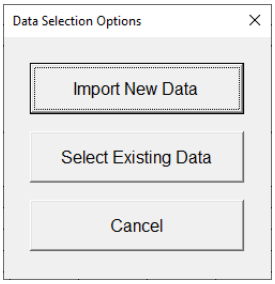
IMPORTANT

In Microsoft Excel, ensure that you enable content.

5 To choose the data for analysis:

a Click **Select data**.

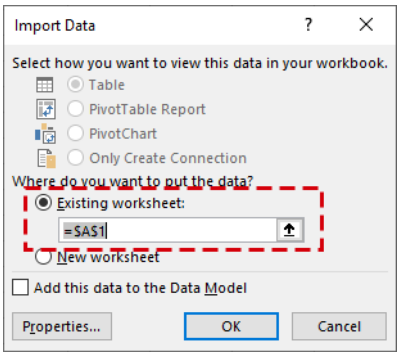
b In the **Data Selection Options** dialog box, click **Import New Data**.



c In the **Import Text File** dialog box, select the text (TXT) data file you want to analyze and click **Import**.

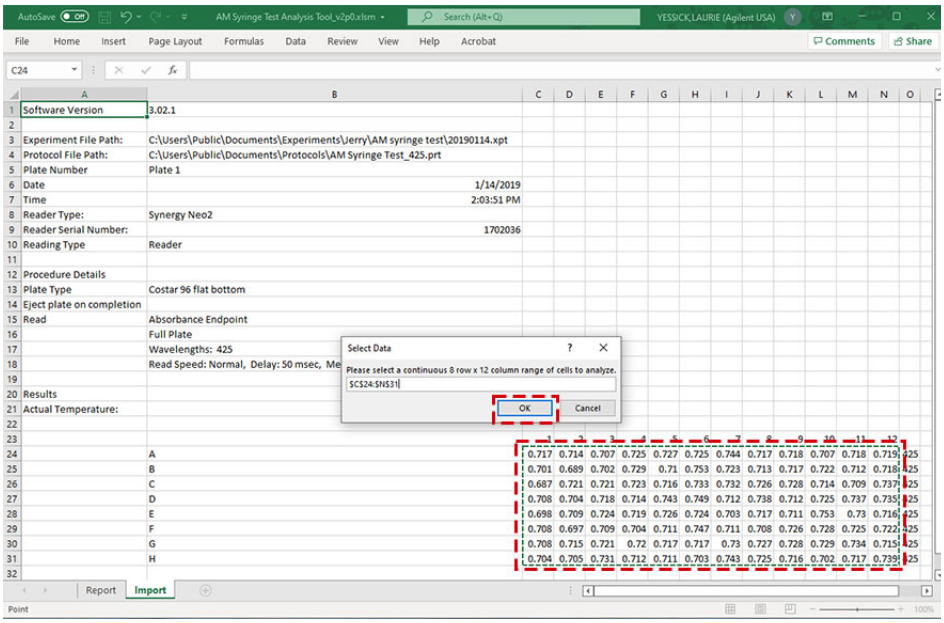
d Follow the instructions in the text import wizard:

- In the **Text Import Wizard Step 1 of 3** dialog box, click **Finish** to accept the default settings.
- In the **Import Data** dialog box, make sure **Existing worksheet** is selected and then click **OK**.

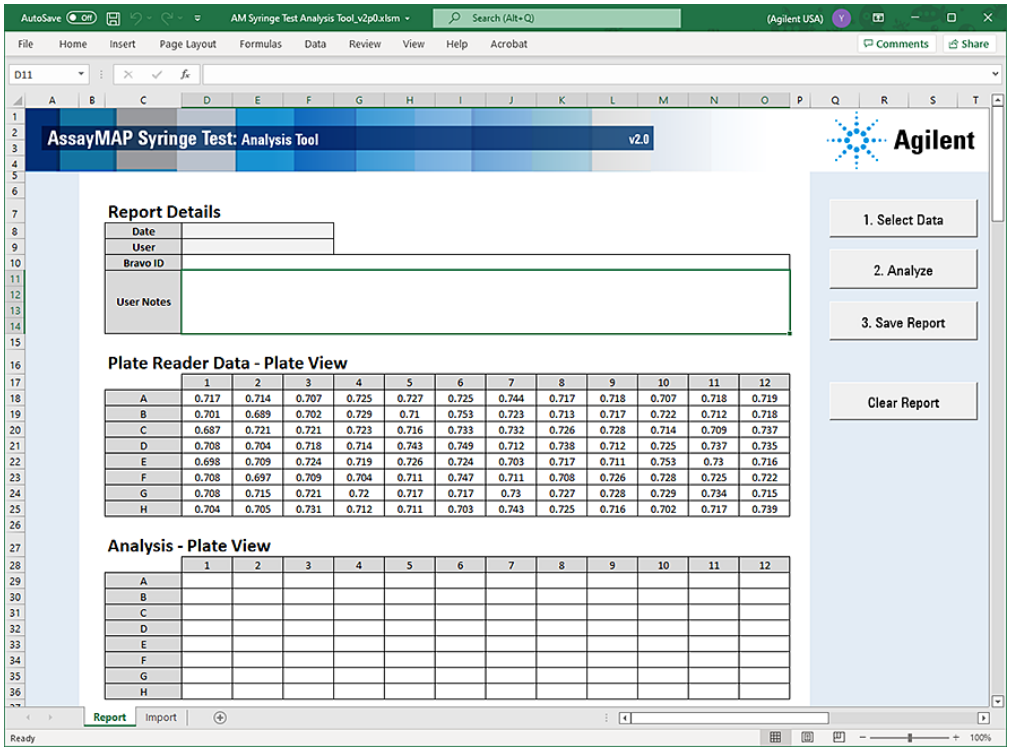


e When the **Select Data** dialog box appears asking you to **Select a continuous 8 row by 12 column range of cells to analyze**, select the 96 cells you want to analyze and click **OK**.

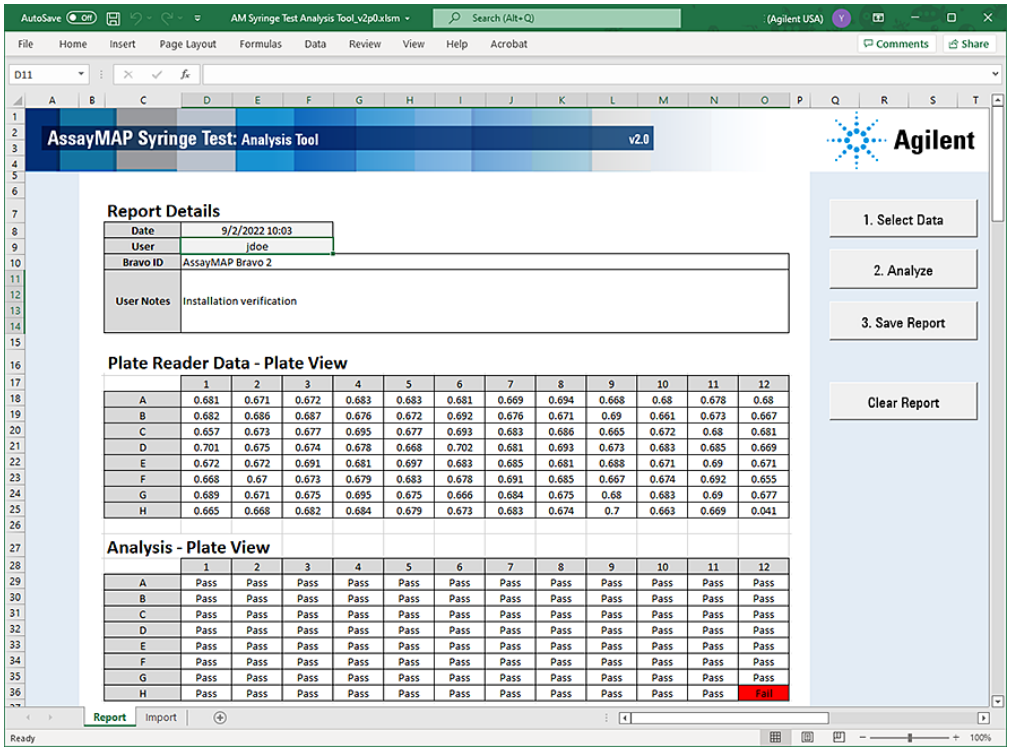
You must select 96 cells, otherwise you will receive a message requesting you to select 96 entries.



The data appears in the Analysis Tool window.



6 Click **Analyze.** The Analysis Tool analyzes the imported data and displays the results in the Analysis - Plate View area.



Failed syringes are indicated by red cells. A list of the failed syringes is located below the analysis results.

Failure List

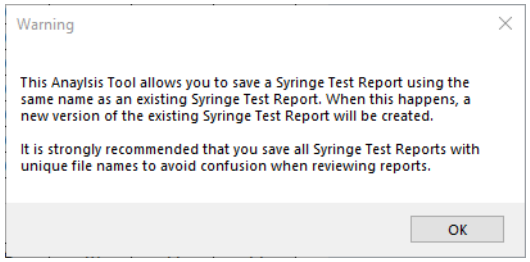
Number	Row	Column	Notes
1	H	12	Complete syringe failure detected. Contact technical support for repair instructions.

To verify that failures are defective syringes, go to "Verifying defective syringes" on page 565.

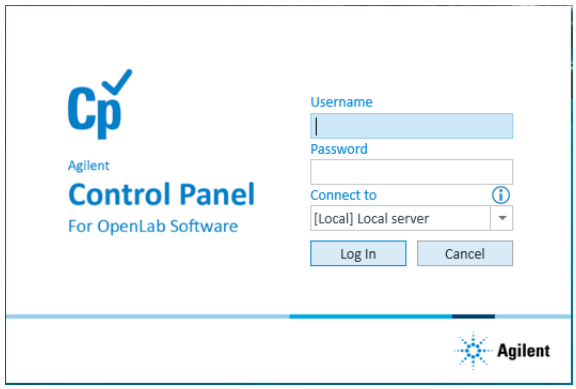
7 Save the results of the test:

a Click **Save Report**.

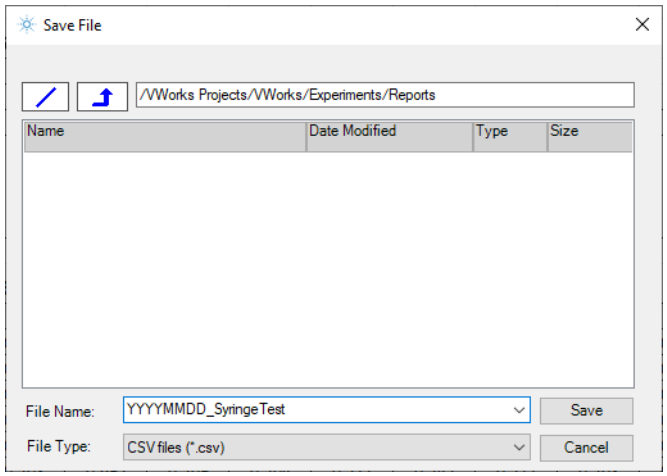
b Read the warning message that opens, and then click **OK**.



c In the Control Panel login window, type your VWorks user name and password, and click **Log In**.



d In the **Save File** dialog box, specify the file name and the storage location:



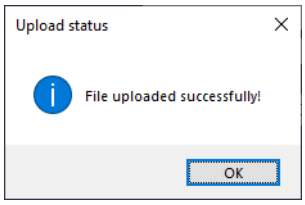
Make sure the file name is unique to avoid creating multiple versions of the Syringe Test report with the same name, for example, YYYYMMDD_Syringe Test

The default Shared Service storage location is

- VWorks Plus:
  … /VWorks Projects/VWorks/Experiments/Reports
- VWorks Standard:
  C:\OLSS Projects\VWorks Projects\VWorks\Experiments/Reports

Click **Save.**

8 Click **OK** when the uploaded successfully message appears.



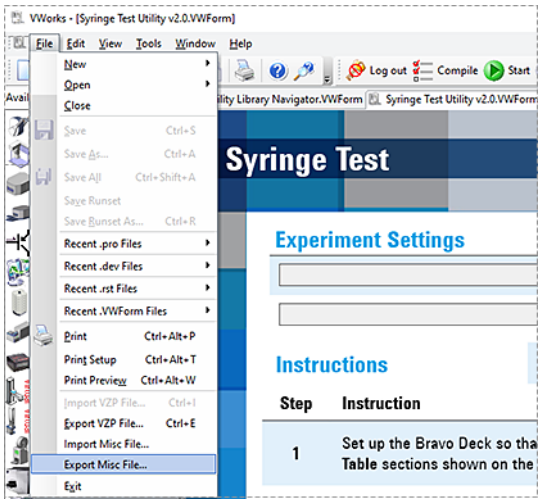
Exporting a Syringe Test report

To create a copy of the report that you can open in Excel, use the following procedure to export the report.

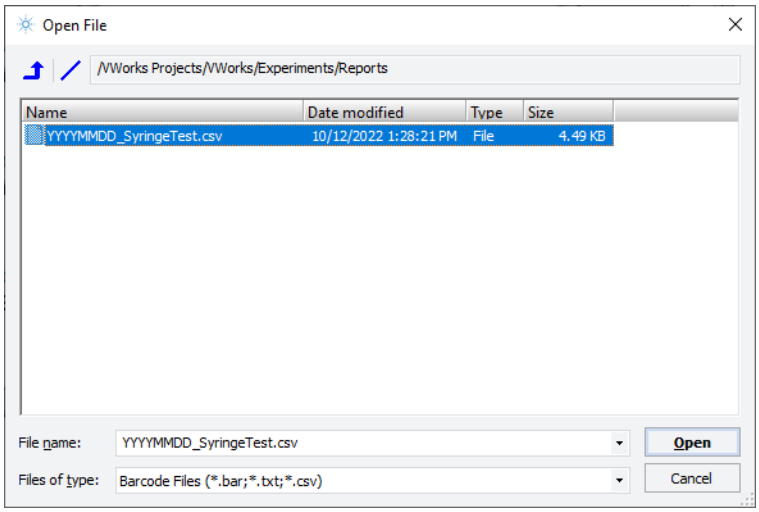
To export a Syringe Test report:

1 In the navigation pane of the Syringe Test form, click Toggle Full Screen to turn off full screen display so that the VWorks menu bar is visible.

2 In the VWorks window, click **File > Export Misc File**.



3 In the **Open File** dialog box, select the report (.csv file) and then click **Open**.



4 In the **Export Miscellaneous File** dialog box, select the destination export location, and then click **Save**.

Verifying defective syringes

Use the following procedure to verify that failed test results indicate defective syringes and not a non-syringe-related problem.

This procedure specifies volume requirements for the default labware selections. If you choose different labware types from the list of options, you should adjust the volumes accordingly.

Before you start

- Check the wash station carboys to ensure:
  - Source carboy has a sufficient supply of source water.
  - Waste carboy has sufficient unused capacity to accept the waste water from the run.
- Prime the wash station by running the Startup protocol. See "Setting up the Startup protocol" on page 576.
- Rotate the rack of test cartridges at location 6 by 180° so that the cartridge that was in the A1 position is now in the H12 position.
- Add 65 mL of water to the remainder of the liquid in the Water Reservoir at deck location 4.
- Add 22 mL of Test Solution to the remainder of the liquid in the Test Solution Reservoir at location 7.

Procedure

To verify defective syringes:

1 Rerun the Syringe Test utility protocol. See "Starting the Syringe Test protocol run" on page 555.

2 Analyze the data. See "Analyzing the results" on page 559.

3 Compare the results to the first test:

- If a syringe fails both tests, the syringe is defective and should be replaced. See "Replacing defective syringes" on page 566 for repair options.
- If a failing test result is not replicated, the syringe is acceptable for use. The initial failing result might have been the result of a non-syringe related problem.

  For example, a faulty cartridge will give a failing result on both the test and retest, but the location of the failure will track with the cartridge. This is why the cartridge rack is rotated by 180-degrees before the verification test.

Replacing defective syringes

To replace damaged syringes, do one of the following:

- Use the AssayMAP Syringe Replacement Kit (part number G5409-68002), and follow the included instructions.
- Contact Agilent Technical Support to schedule a service call.

If you continue to use the AssayMAP Bravo Platform while awaiting repair, do not use data obtained from the defective syringe.

Syringe Wash v3.0 User Guide

The Syringe Wash v3.0 utility enables you to conduct up to 6 sequential syringe washes, using up to 6 different wash solutions (plus, water in the wash station). You select which buffer to use for each of the 6 syringe washes. The same wash buffer can be selected for multiple washes.

Before you start

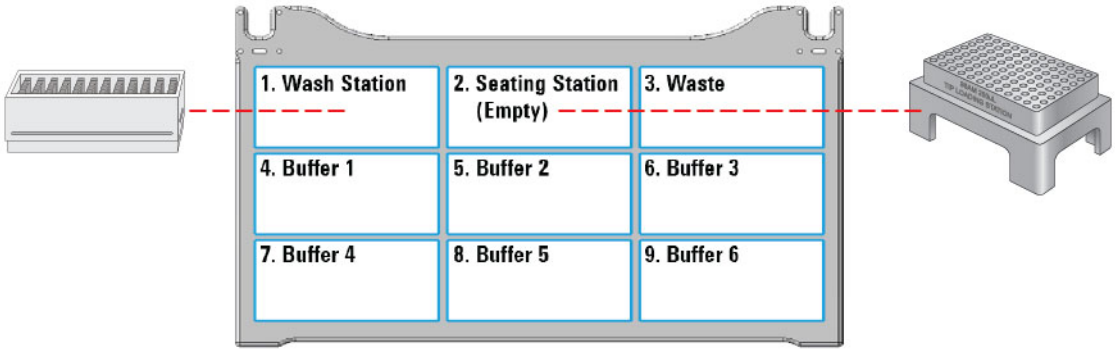
Labware

The following table provides a complete list of labware options and the corresponding deck locations.

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

IMPORTANT Make sure the 96AM Cartridge & Tip Seating Station at deck location 2 is empty. (The station should not contain cartridges or pipette tips.)

Figure Labware locations on the Bravo deck (top view)



Labware options (deck locations 3–8)	Manufacturer part number*
1.2mL Deep-Well PolyPro Clear Plate	ABgene AB-1127
96-Well U-Bottom PolyPro Clear Plate	Greiner 650201
12-Column Low-Profile Reservoir	Agilent 201280-100
8-Row Low-Profile PolyPro Reservoir	Agilent 201282-100

Labware options (deck locations 3–8)	Manufacturer part number*
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottom	Agilent 201254-100
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP
96 V11 Manual Fill Reservoir	Agilent G5498B#049
*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.	

Reagents

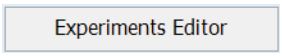
An wash solution of 50 mM NaOH has been effective for washing the syringes between most protein and peptide sample preparation runs. The Syringe Wash utility provides up to 6 washes so that you can use multiple washes using solutions that cover different chemical spaces to address contaminates that are difficult to remove from the syringes.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

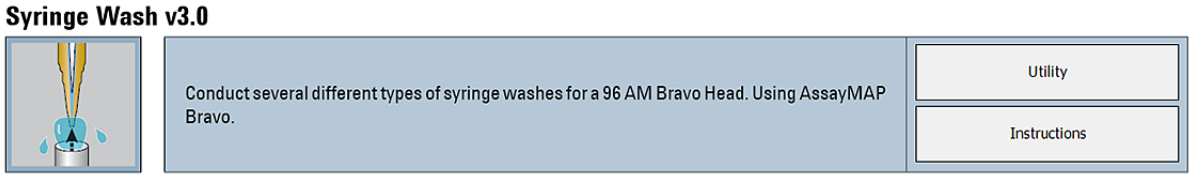
Setting up the protocol

Before starting the protocol, make sure the appropriate selections and values are specified in the Syringe Wash utility.

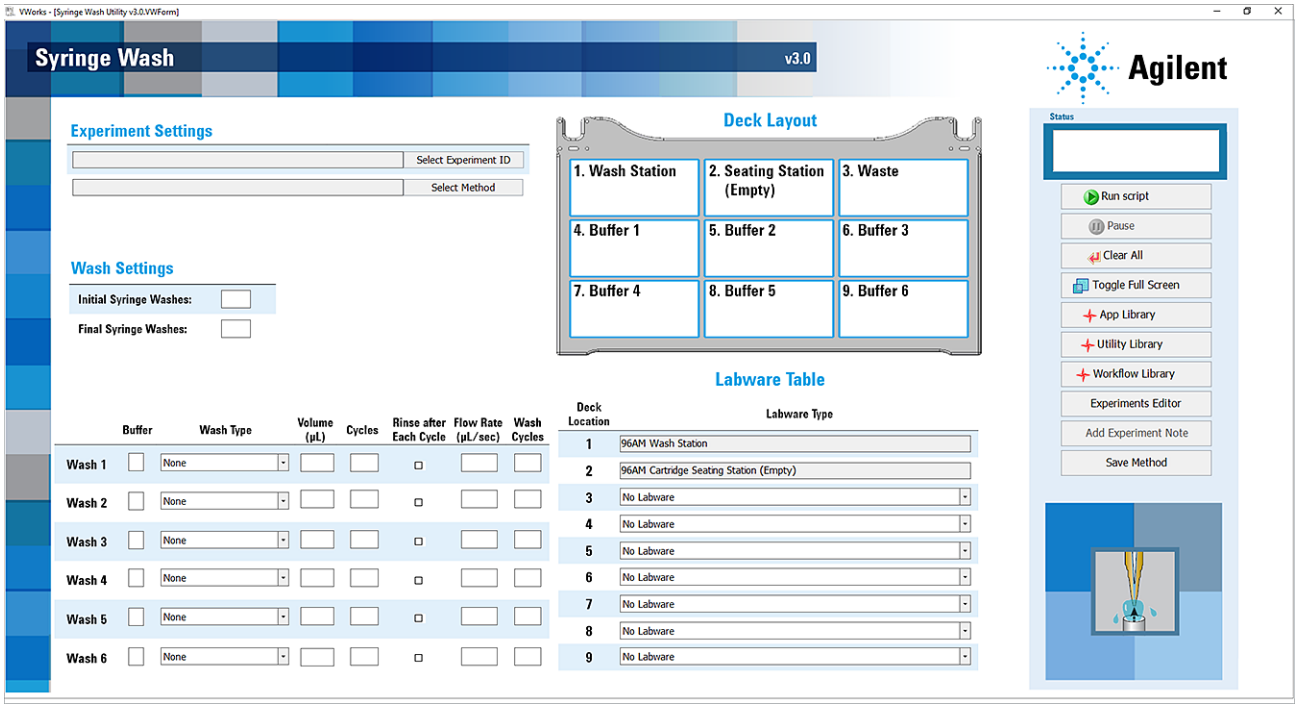
To set up the protocol:

1 Open the **Utility Library**.

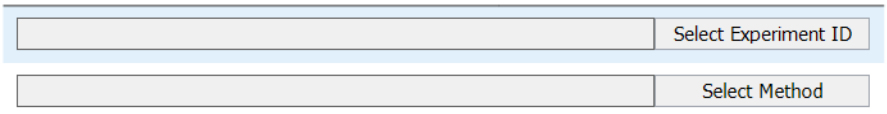
2 Locate **Syringe Wash**, and then click **Utility**.



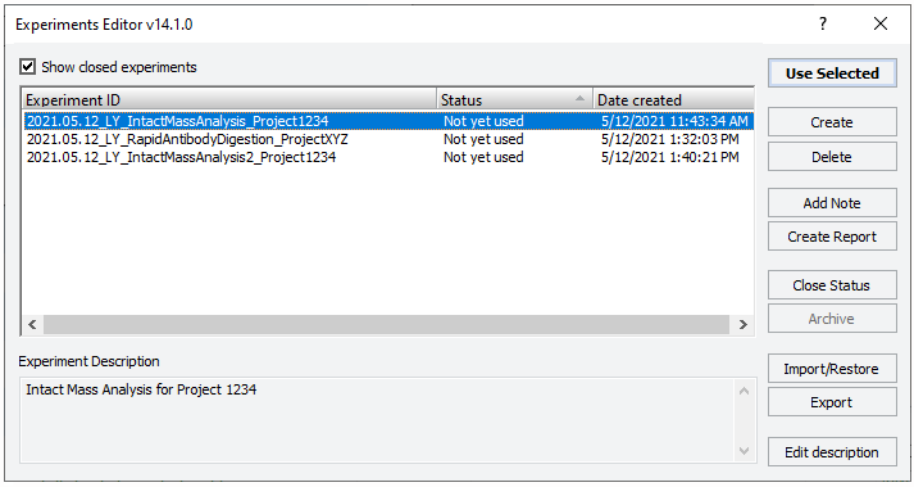
The Syringe Wash utility opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Syringe Wash run" on page 572.
- To create or modify a method, proceed to step 6.

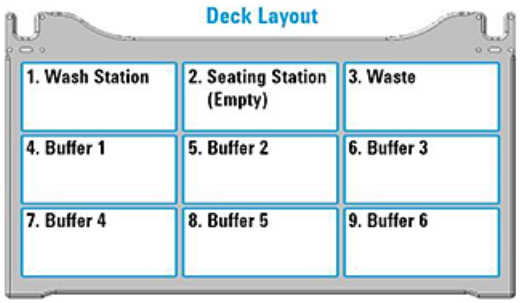
VWorks Plus. Administrator or technician privileges are required to create and modify methods.

6 In the **Application Settings** area, specify the following settings for the run:

a Under **Wash Settings**, specify the number of Syringe Washes at the wash station at the beginning and end of the run:

- **Initial Syringe Washes**. The number of standard Internal Syringe Washes to be conducted using the wash station at deck location 1, before starting any of the syringe washes.
- **Final Syringe Washes**. The number of standard Internal Syringe Washes to be conducted using the wash station at deck location 1, after completion of all the syringe washes.

b Set the following properties for each syringe wash. You can conduct up to six washes:

Property	Description
Buffer	Set the deck location of the Buffer reservoir for the given syringe wash. The same deck location can be used for multiple syringe washes.  Defaults: • Wash 1: Buffer 1 (deck location 4) • Wash 2: Buffer 2 (deck location 5) • Wash 3: Buffer 3 (deck location 6) • Wash 4: Buffer 4 (deck location 7) • Wash 5: Buffer 5 (deck location 8) • Wash 6: Buffer 6 (deck location 9)
Wash Type	The type of wash: • **NONE**. Select this option to specify no wash. If you select NONE for any of the 6 syringe washes, its corresponding syringe wash will be skipped. • **Cycle in Reservoir**. Select this option to conduct aspirate-and-dispense cycles in the designated wash buffer location. This option will not move the wash solution out of its original location. • **Discard – Waste Plate**. Select this option to aspirate from the specified Wash Buffer reservoir and dispense into the Waste Plate at deck location 3. **IMPORTANT** Ensure that the settings you specify will not overflow the capacity of the labware designated as the Waste Plate. • **Discard – Wash Station**. Select this option to aspirate from the specified Wash Buffer reservoir and dispense into the wash station waste sump. Default: • Wash 1: Cycle in Reservoir • Wash 2 – Wash 6: None

Property	Description
Volume (µL)	The volume to be aspirated into the syringes and dispensed for each wash cycle. Range: 0–250 Default: 250
Cycles	The number of aspirate-and-dispense cycles to be conducted for each syringe wash. Range: 0–60 Default: 5
Rinse after Each Cycle	To wash the syringes at the wash station after each wash cycle in this wash, select the check box. Otherwise, clear the check box. Default: Not selected
Flow Rate (µL/sec)	The volumetric speed of the pipetting axis (*w*-axis) during each wash cycle. Range: 0–300 Default: 200 *Note:* Although the AssayMAP flow rate is normally expressed in µL/min, use µL/sec on this form.
Wash Cycles	The number of Internal Syringe Washes at the wash station to be conducted after all the wash cycles are completed for the given wash. Range: 0–10 Default: 0

7 In the **Labware Table**, select that labware you are using:

a **Deck Location 3**. Select the labware for the Waste plate.

b **Deck Location 4**–**9**. Select the labware for the Buffer at each deck location that you are using for this wash method.

Note: The wash method can include up to six syringe washes.

8 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

VWorks Plus. You must save the method before you can run it.

Starting the Syringe Wash run

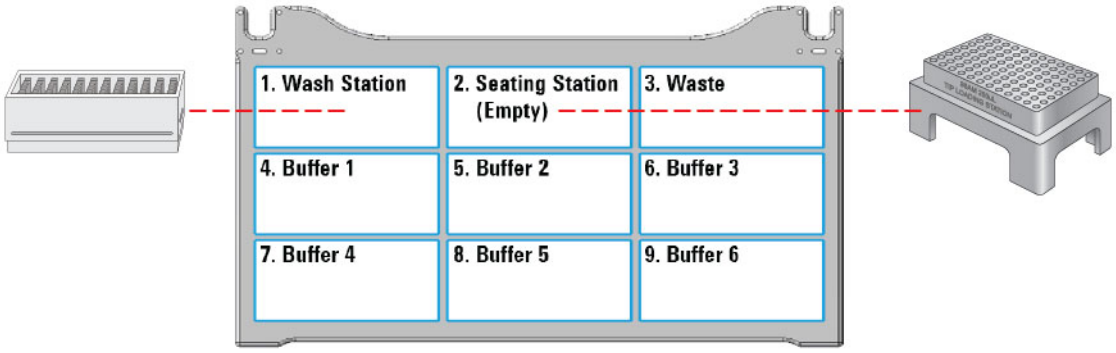
WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

Make sure the volume of solution in the labware is sufficient for the overage requirement plus the parameters selected on the form. For volume overage requirements for a given labware type, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Make sure the labware are properly seated on the Bravo deck.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

IMPORTANT Ensure that the volume parameter specified in the form will not result in the overflow of waste at deck location 3.

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box in the upper right corner of the form.

When the run is finished, remove the labware from the deck.

WARNING **To stop a run in an emergency, use the emergency stop pendant.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

System Startup/Shutdown v3.0 User Guide

The System Startup/Shutdown utility consists of two protocols:

- **Startup**. Prepares the AssayMAP Bravo Platform for use by:
  - Initializing the device if this is the first time the Startup utility is run after powering up the AssayMAP Bravo Platform.
  - Dispensing any remaining liquid in the syringes to the wash station.

    Note: The *w*-axis (aspirate/dispense axis) is the up-and-down motion of the piston. The *w*-axis initialization results in emptying the syringes.
  - Priming the wash station lines.
  - Washing the syringes the specified number of wash cycles.

  You should run Startup when you first start up the AssayMAP Bravo Platform, before running an AssayMAP Bravo application or utility if the Shutdown procedure was performed after the last AssayMAP run, or to return the AssayMAP Bravo to a known state.
- **Shutdown**. Prepares the AssayMAP Bravo Platform for idle time by:
  - Washing the syringes the specified number of wash cycles.
  - Aspirating 200 µL of Syringe Storage Liquid (water or a user-defined solution) into the syringes. The Syringe Storage Liquid prevents residual reagents from forming salts and corroding the syringe seals.

  You should run Shutdown after every application protocol run, if the AssayMAP Bravo Platform will not be in use for at least 1 hour, and before turning off the device.

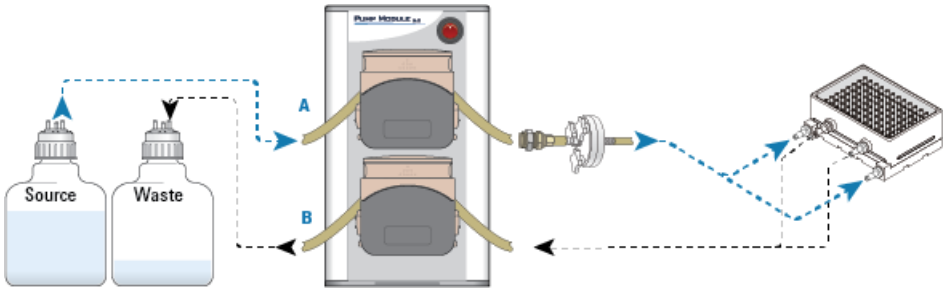
Before you run the Startup protocol

Before running the Startup protocol:

1 Check the liquid levels of the source and waste bottles for the wash station. Fill the source bottle and empty the waste bottle, as required.

Verify that the flip-top cover on each pump head is closed and that the tubing connections are secure.

Figure Wash station fill line (A) and empty line (B)



2 Turn on the Pump Module and Peltier Thermal Station Controller.
3 Ensure the following items are in position on the AssayMAP Bravo deck:
   - 96AM Wash Station or the later model 96 Channel Wash Station at deck location 1
   - Empty 96AM Cartridge & Tip Seating Station at deck location 2
4 Turn on the AssayMAP Bravo Platform.
5 Open the **System Startup/Shutdown** utility.

Before you run the Shutdown protocol

Before running the Shutdown protocol:

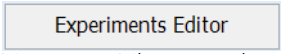
1 If more rigorous syringe washing is desired, run the Syringe Wash utility before running the Shutdown protocol. For instructions, see "Syringe Wash v3.0 User Guide" on page 567.
2 Check the liquid levels of the source and waste bottles for the wash station. Fill the source bottle and empty the waste bottle, as required. Verify that the tubing connections are secure.
3 Ensure the following items are in position on the AssayMAP Bravo deck:
   - 96AM Wash Station or the later model 96 Channel Wash Station at deck location 1
   - Empty 96AM Cartridge & Tip Seating Station at deck location 2
   - *Optional*. Acceptable reservoir containing an appropriate Syringe Storage Liquid at deck location 7
4 Open the **System Startup/Shutdown** utility.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

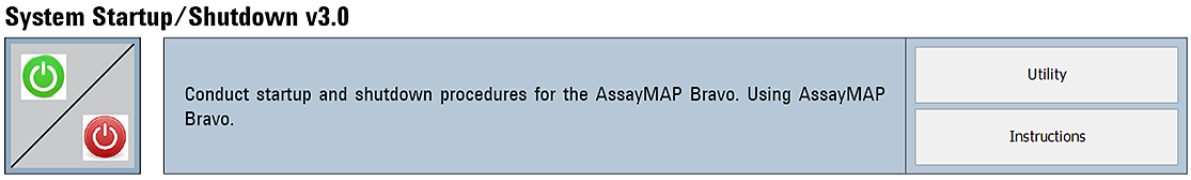
Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

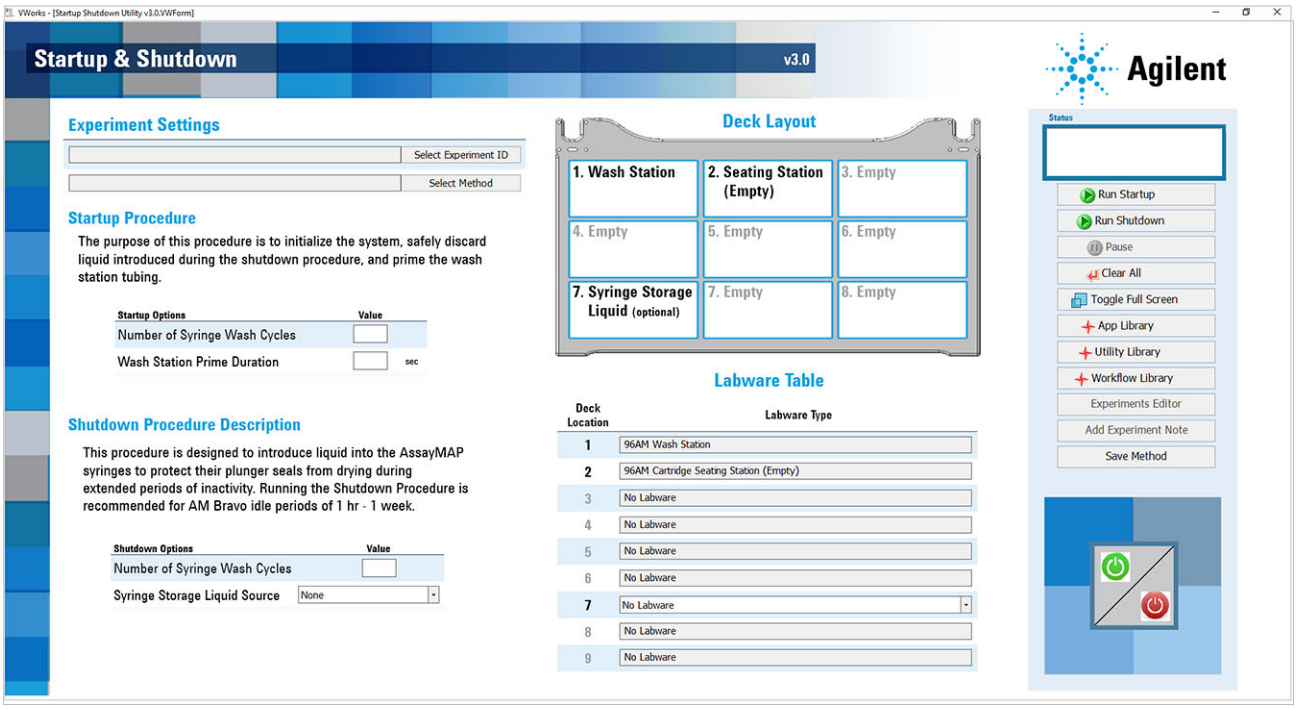
Opening the System Startup/Shutdown utility

To open the Startup/Shutdown utility:

In the **Utility Library**, locate the **System Startup/Shutdown** utility, and then click **Utility**.



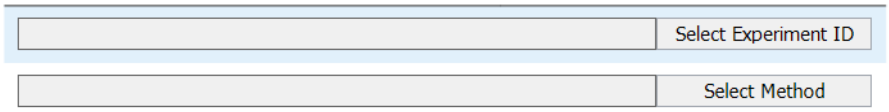
The System Startup/Shutdown utility opens.



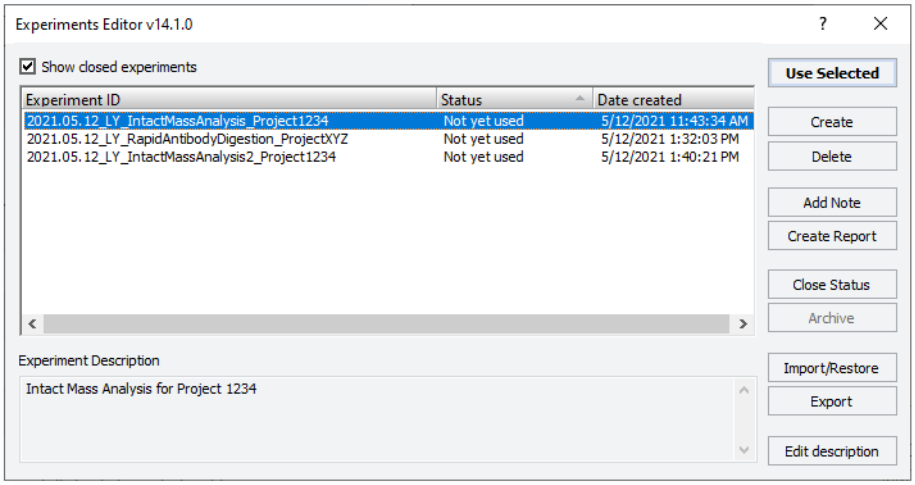
Setting up the Startup protocol

To set up the Startup protocol:

1 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



2 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

3 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Startup run" on page 578.
- To create or modify a method, proceed to step 4.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

4 In the **Startup Options** area of the form, enter the values for the following properties:

Property	Description
Number of Syringe Wash Cycles	Specifies the number of syringe wash cycles that will be conducted at the wash station at deck location 1. Default: 3 Range: 0–10
Wash Station Prime Duration (sec)	Specifies how long (seconds) to run the wash station pumps in order to prime the tubing lines and wash station chimneys. If the tubing lines are dry, set this value to 60 seconds. Default: 10 Range: 0–300

5 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

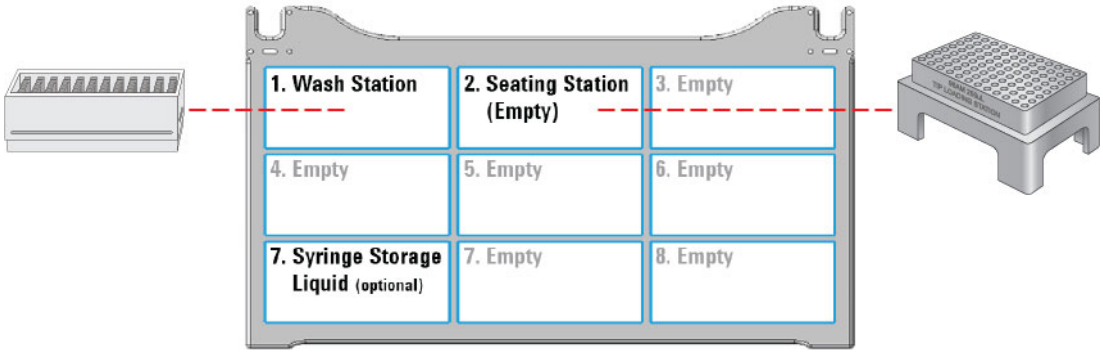
VWorks Plus. You must save the method before you can run it.

Starting the Startup run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

1 Ensure that the accessories are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

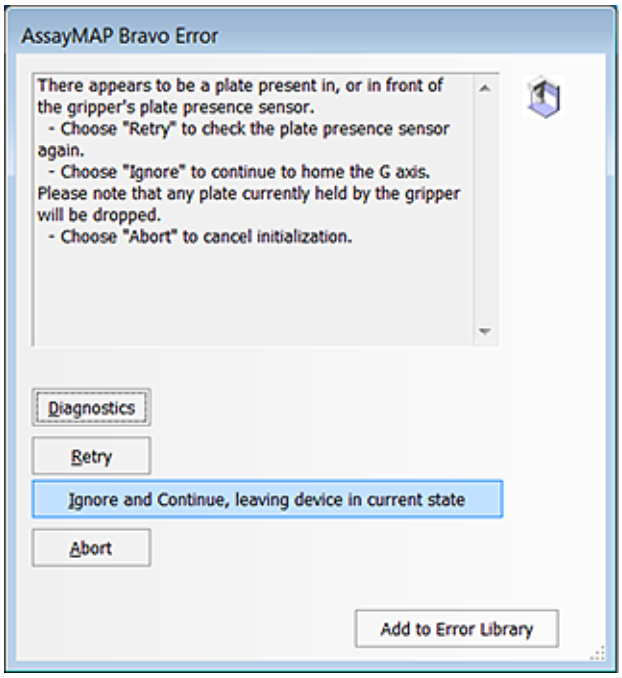


CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

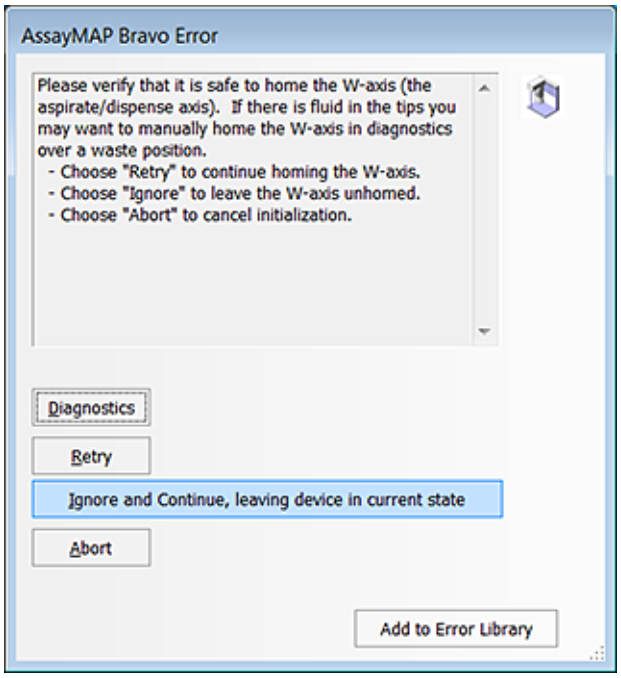
2 Click Run Startup.

- If this is the first time the Startup utility has been run after powering up the AssayMAP Bravo Platform, the device initialization process begins. Proceed to step 3.
- If the platform is already initialized, skip to step 5.

3 When the following message appears, click **Ignore and Continue, leaving device in current state** to proceed to initialize the grippers.



4 When the following message appears, click **Ignore and Continue, leaving device in current state** to delay initializing the *w*-axis. The *w*-axis will automatically initialize in a later step when the Bravo 96AM Head is positioned over the wash station at deck location 1.



When the initialization process is finished, the orange lights on the AssayMAP Bravo Platform light panel flicker briefly and then begin to flash.

The Bravo 96AM Head does the following:

- Moves to deck location 2 and parks any cartridges remaining on the head.

- Moves to the wash station at deck location 1 and dispenses any liquid remaining in the syringes.
- Washes the syringes the specified number of times.

The priming step begins after the syringe wash.

5 During the priming step, ensure that fluid flows out of the top of each chimney. Check for any damaged chimneys in the wash station.

Click **Continue** to complete the Startup protocol.

Troubleshooting problems	Probable cause and solution
Fluid does not flow out of a chimney.	A trapped air bubble can block the fluid flow out of a chimney. ***To clear the bubble:*** 1 Push the air out of a 1000 µL pipette with a pipette tip attached. Form a seal between the top of the problematic chimney and the 1000-µL pipette tip, and then draw the air and then water through the chimney at a medium speed. 2 Run the Startup protocol again to determine if the bubble was successfully removed. 3 Repeat if necessary.
Chimney deformity or irregularity	Chimney irregularities can result from a collision between the head and the wash station. For additional troubleshooting guidelines or to replace damaged chimneys, see the *96 Channel Wash Station Maintenance Guide*.

When all the chimneys are flowing evenly, the device is ready for operation.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

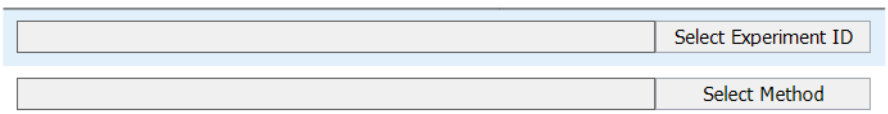
To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

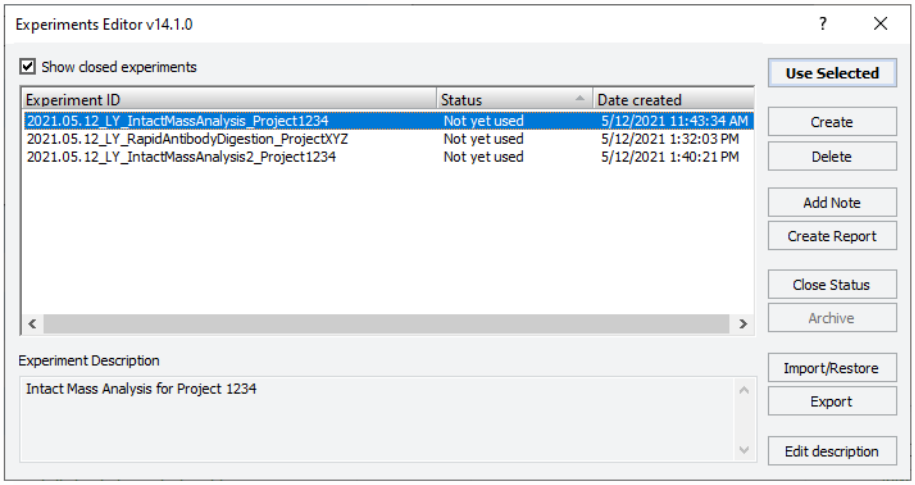
Setting up the Shutdown protocol

To set up the Shutdown protocol:

1 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



2 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

3 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

- To run the selected method, go to "Starting the Shutdown run" on page 582.
- To create or modify a method, proceed to step 4.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

4 In the **Shutdown Options** area of the form, enter the values for the following properties:

Property	Description
Number of Syringe Wash Cycles	Specifies the number of syringe wash cycles to conduct at the wash station at deck location 1. Default: 3 Range: 0–10
Syringe Storage Liquid Source	Determines the location from which to aspirate the syringe storage liquid. The options are: • 96AM Wash Station. The liquid is aspirated from the chimneys of the wash station at deck location 1. • **Syringe Storage Liquid**. The liquid is aspirated from the labware that you specified at deck location 7. For this option, ensure that you: – Select the labware type for deck location 7 in the **Labware Table** area. – Provide the minimal volume overage required for the labware type being used, plus 200 µL per syringe. For the labware overage requirements, see the *Labware Reference Guide* (Workbench Literature Library page).

5 To save the method:

a Click Save Method.

b In the **Save File As** dialog box, type the file name and click **Save**.

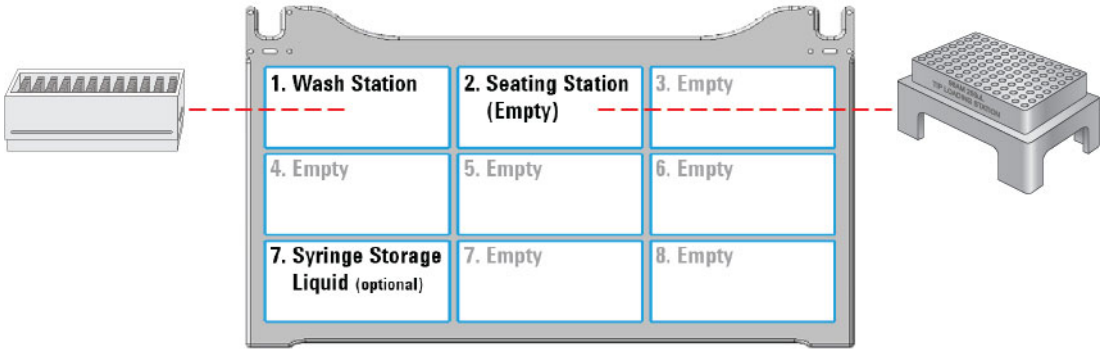
VWorks Plus. You must save the method before you can run it.

Starting the Shutdown run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the Shutdown run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.



CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Shutdown. The shutdown process begins.

3 Observe the AssayMAP Bravo head as it does the following:

- Moves to deck location 2 and parks any cartridges remaining on the head.
- Moves to the wash station at deck location 1 and dispenses any liquid remaining in the syringes.
- Washes the syringes the specified number of times.
- Aspirates the 200 µL of Syringe Storage Solution into the syringes from the specified location.

4 When the protocol finishes:

a Remove all labware from the AssayMAP Bravo deck.

b Discard the excess Syringe Storage Liquid, if necessary.

c Empty the waste bottle.

WARNING **Make sure you discard the chemical waste according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

5 If the AssayMAP Bravo Platform will remain unused overnight:

- **a** Exit the Protein Sample Prep Workbench.
- **b** Shut down and turn off the computer.
- **c** Turn off the Bravo Platform.
- **d** Turn off the accessories.
- **e** At the Pump Module, lift the flip-top cover on each pump head to prevent flattening the tubing and to help maximize the tubing life.

Automation movements — Startup protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Startup protocol using the default protocol method. Changing the selections or parameters will alter the movements.

Head moves to deck location...	Action
2	Parks any cartridges that may have been mounted on the head from a protocol that had been previously aborted.
1	Dispenses any liquid remaining in the syringes into the wash station.
1	Washes the syringes the specified number of times.
2	Moves the head to a safe height above deck location 2 to enable user observation of the wash station at deck location 1.
1	Primes the wash line and fills the wash station with liquid from the wash buffer reservoir for the specified duration.
1	Moves the head to a safe height above deck location 1.

Automation movements — Shutdown protocol

This section describes the basic movements of the AssayMAP Bravo Platform during the Shutdown protocol using the default method settings. Changing the selections or parameters will alter the movements.

Protocol steps	Head moves to deck location...	Action
	2	Parks any cartridges that may have been mounted on the head from a protocol that had been previously aborted.
	1	Dispenses any liquid remaining in the syringes into the wash station.
	1	Washes the syringes the specified number of times.

Protocol steps	Head moves to deck location...	Action
	1	Fills the wash station chimneys with DI water.
	1	Aspirates 200 µL of DI water from the wash station into the syringes.
	or	
	7	Aspirates 200 µL of Syringe Storage Liquid from the reservoir at deck location 7 into the syringes.
	1	Moves the head to a safe height above deck location 1.

16 Normalization v3.0 User Guide

This chapter contains the following topics:

Utility description

Normalization v2.0. This utility enables you to convert a sample plate containing samples of various concentrations into a sample plate containing samples of a uniform concentration. The utility cycles through each sample, one at a time, combining a calculated volume of each sample with an appropriate volume of diluent.

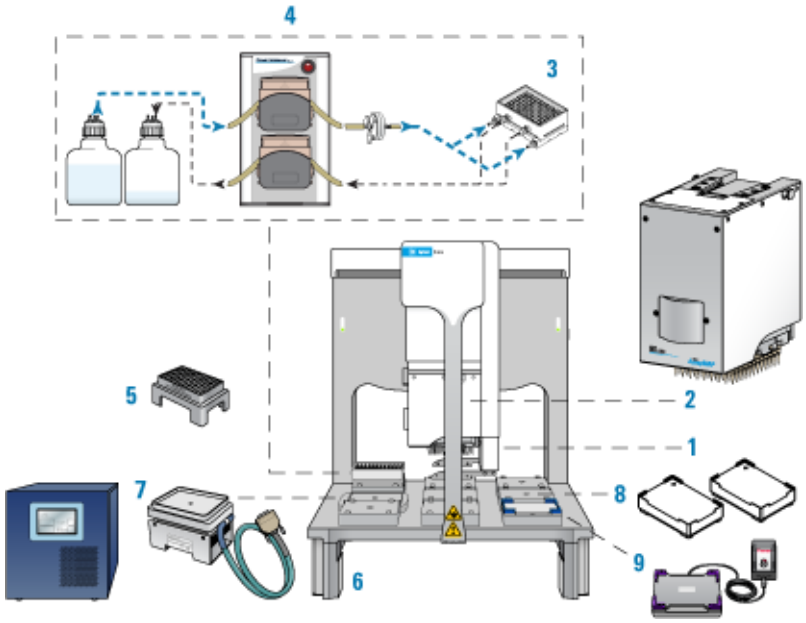
Before you start

This topic lists the required hardware, software, labware, and solutions for running the Normalization protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The AssayMAP Bravo Platform is required for running the AssayMAP protocols. The following figure and table describe the platform components.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Bravo Risers, 14.6 cm
7	Peltier Thermal Station with STC controller
8	Plate Risers, 2.84 cm (two)
9	Orbital Shaking Station with Control Unit

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent VSpin Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

Labware

The Normalization protocol requires the following labware:

- Full tip box of 96 250-µL pipette tips, Agilent 19477-002
- Labware for the diluent reservoir, initial samples plate, and normalized samples plate

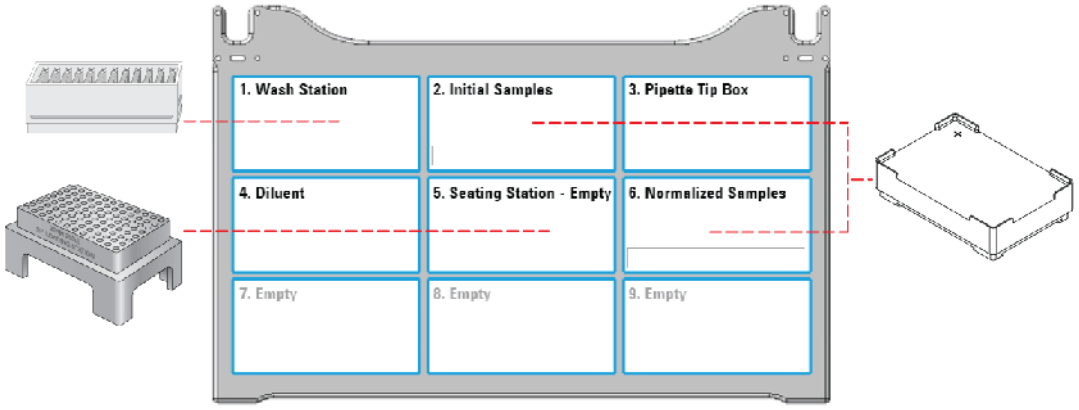
The following table provides a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Using different pipette tips, such as filtered tips, will result in equipment damage. Ensure that you use the standard 250-µL pipette tip (Agilent 19477-002).**

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Accessory and labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	2, 6
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	2, 6
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	2, 6
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	2, 6
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	2, 6
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	2, 6
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	2, 6
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	2, 6
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	4
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	4
96 V11 Manual Fill Reservoir	Agilent G5498B#049	4
Reservoir, Axygen Scientific RES-SW96-LP, 86mL pyramid bottom	Axygen Scientific RES-SW96-LP	4

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

The unusable volume (also known as *dead* volume) is the volume that cannot be reliably aspirated from the microplate well due to pipetting limitations that arise from differences in well-bottom geometry. The following table provides some general guidelines on how different microplate geometries can affect this value.

Table Guidelines on how microplate geometry can affect volume requirements

Well-bottom geometry	Examples	Unusable volume expectations
Flat bottom	Greiner 675801	Generally, the worst plates for minimizing dead volume. Liquids are attracted to the intersection of the plate bottom and side walls.
Round (U) bottom	Greiner 650201, Greiner 650207	Generally, good options for minimizing dead volume. A round bottom forces liquid to collect in the center of the well at a medium rate.
V-bottom and PCR	• V-Bottom: Greiner 651201, Corning Costar 3363 • PCR Plate: Eppendorf 30129300, Greiner 652270	Generally, the best plates for minimizing dead volume. Steep side walls force liquid to collect in the center of the well, making it easy to access.

For volume overage recommendations by labware type, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Solutions

The Normalization utility requires the following solutions:

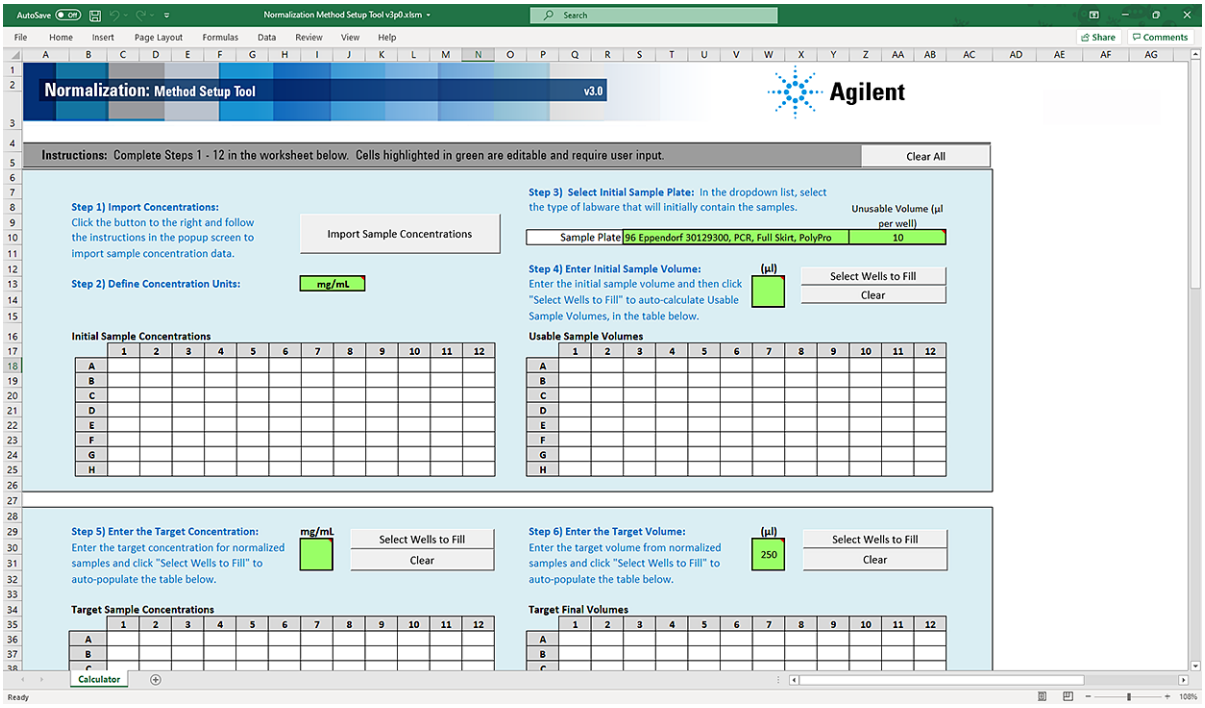
- Diluent to adjust the sample concentration
- Purified water for the wash station reservoir (carboy)

Setting up a Normalization method

The Method Setup Tool is a Microsoft Excel-based tool that steps you through the process of creating and saving a method file that contains all the information required to run the Normalization protocol on the AssayMAP Bravo Platform. This tool uses formulas to calculate volume requirements for the samples and diluent based on your input.

Note: When you select a method in the Normalization utility, the form displays the corresponding labware selections and diluent preparation instructions.

Figure Normalization Method Setup Tool



Before you start

Ensure that you have a Microsoft Excel file or a delimited text file (.csv or .txt) that contains the sample concentration values to be imported. The file should contain an 8-by-12 array of values, where

- Each row of the 8-by-12 array represents a row in the 96-well microplate.
- Each column of the 8-by-12 array represents a column in the 96-well microplate.
- An empty cell or a value of 0 is acceptable.

Figure Example of an Excel file with an 8-by-12 grid numbers

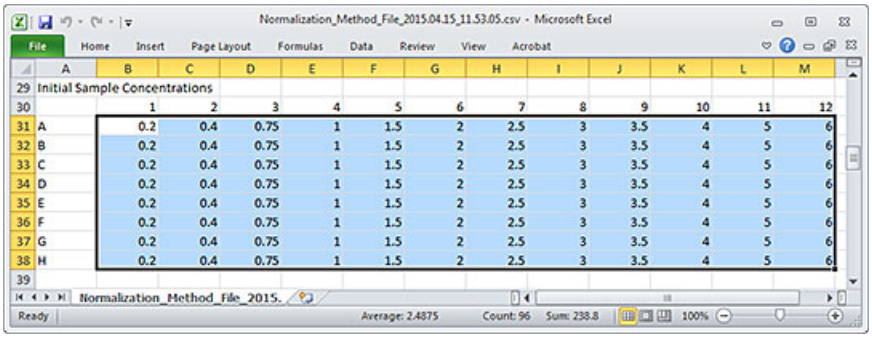
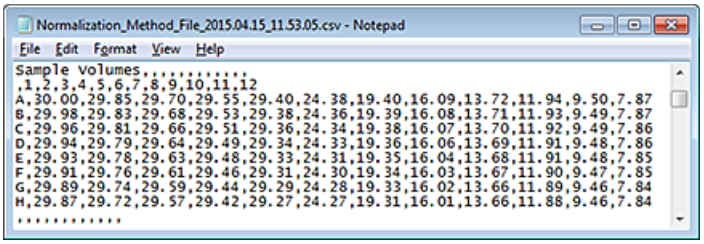


Figure Example of a csv file with an 8-by-12 grid numbers



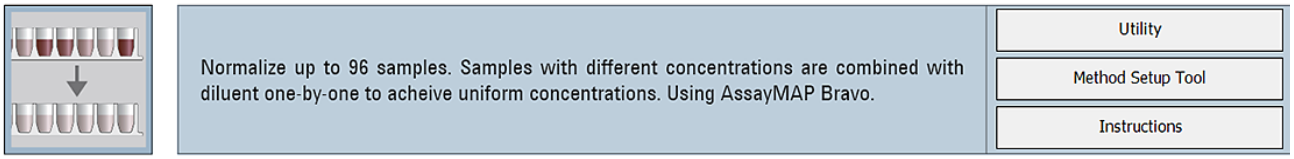
Opening the Method Setup Tool

You can open the tool from the Utility Library or from the Normalization form.

To open the Method Setup Tool:

1 In the **Utility Library**, locate the **Normalization** banner.

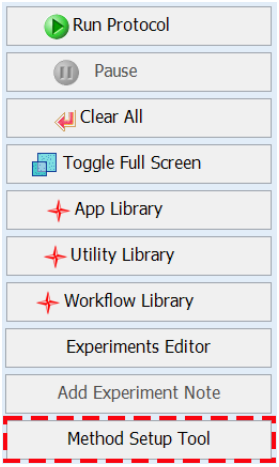
Normalization v3.0



2 Click one of the following buttons:

- **Method Setup Tool**. Microsoft Excel starts and displays the Method Setup Tool.
- **Utility**. The Normalization utility opens.

  In the navigation pane, click **Method Setup Tool**. Microsoft Excel starts and displays the Method Setup Tool.



IMPORTANT In Microsoft Excel, ensure that you enable content.

Overview of steps in Method Setup Tool

The Method Setup Tool has 13 distinct steps. The following table provides an overview of the steps.

Table Overview of steps in Method Setup Tool

Step		Description
Defining Initial Samples plate (steps 1–4)		
1	Import concentrations	Click **Import Sample Concentrations**, and then import the values for the known concentrations in your sample plate from a file (.xls, .xlsx, .csv, or .txt) or enter the values manually.
2	Define Concentration Units	Enter the concentration units in the green box. *Note:* This input is only a reminder. It has no impact on the subsequent calculations.
3	Select Initial Samples Plate	Click the green **Sample Plate** box, and then select the labware from the list. The setup tool uses the value that appears in the **Unusable Volume** box (also known as *dead* volume) to calculate the usable sample volume. You can change the unusable volume manually, if desired, as the default value is a conservative estimate.
4	Enter Initial Sample Volume	Type the initial sample volume in the green **(µL)** box, and then select the array of wells for which this value is correct in the **Usable Sample Volumes** plate grid. The setup tool automatically enters the usable sample volume (that is, the initial sample volume minus the unusable volume) in the **Usable Sample Volumes** plate grid. Repeat step 4 until you have specified the volume for all the wells in the Sample plate that contain a sample.

Step		Description
Defining normalization targets (steps 5–6)		
5	Enter the Target Concentrations	Type the target concentration in the green box, click **Select Wells to Fill**, and then select the array of wells to be filled in the **Target Sample Concentrations** plate grid. Repeat step 5 until you have specified the concentrations for all the wells.
6	Enter the Target Volume	Type a value in the green **(µL)** box that you want to apply to multiple wells of the microplate, and then click **Select Wells to Fill**. Select the array of wells to be filled in the **Target Final Volumes** plate grid. Repeat step 6 until you have specified the volumes for all the wells.
Calculating results and dealing with exceptions (steps 7–8)		
7	Calculate Normalization Volumes	Click **Calculate Volumes**. The calculated volumes (µL) display in the **Diluent Volumes to Use** and the **Sample Volumes to Use** plate grids.
8	Manage Calculation Exceptions	Click **Check Exceptions**. In the **Manage Exceptions** dialog box, select the option that is appropriate for each sample that is flagged as problematic. The setup tool wizard guides you through the process.
Finalizing Normalization method setup (steps 9–13)		
9	Define Remaining Labware Types	Click the green **Normalized Plate** box, and then select the labware from the list. The **Maximum Volume (µL per well or channel)** for the selected plate is automatically displayed. *Note:* The final normalized sample volume must be equal to or less than the well volume of the selected plate type, or the Maximum Volume (µL per well or channel) cell will turn red. Click the green **Diluent Reservoir** box, and then select the labware from the list.
10	Select the Number of Mix Cycles	Click the green **mix cycles** box, and then select the value from the list. *Note:* The number of mix cycles is dependent on the volume, viscosity, and size of molecules in the solution being mixed.
11	Input Evaporation Correction Factor	Type a value in the green **(%)** box for the correction factor, and then click **Apply Correction Factor**. The setup tool automatically adjusts the values in the Final Diluent Volumes and Final Sample Volumes plate grids.
12	Check Volume Required for Diluent Reservoir	Review the **Diluent required for Diluent Reservoir** area at the bottom of the worksheet. If a red highlight appears on the **Total to fill Reservoir** value, the diluent volume plus required overage is more than the selected labware can hold. In this case, select a different labware option for the **Diluent Reservoir** in step 9. Make a note of the **Total to fill Reservoir** volume. You will use this value to prepare the diluent reservoir for the Normalization run. This volume includes the required overage.
13	Create a Normalization Method File	Click **Create Method File**. After you save the method, the Method Setup Tool closes. When you select the method in the Normalization utility, the form displays the corresponding labware selections and diluent preparation instructions. Follow the instructions that appear in the form to prepare the diluent and sample plates.

Step 1) Import Concentrations

To import the values for the known concentrations into the initial samples plate, use one of the following procedures:

- Importing values from an Excel file
- Importing values from a text file

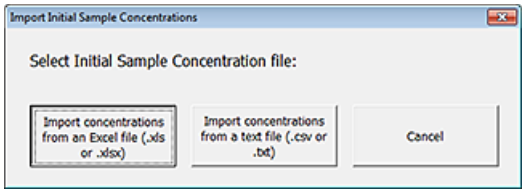
Note: Empty cells or cells with a value of 0 are acceptable.

Importing values from an Excel file

To import the values from an Excel file (.xls or .xlsx):

1 To clear any data from the Normalization Method Setup Tool, click **Clear All** (upper right corner).

2 Click **Import Sample Concentrations**. The Import Initial Sample Concentrations dialog box opens.



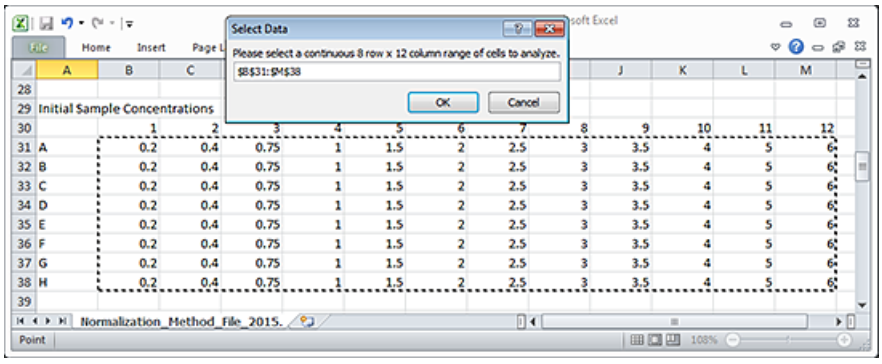
3 Click **Import concentrations from an Excel file (.xls or .xlsx)**.

4 In the **Open Excel File** dialog box, select the file, and then click **Open**.

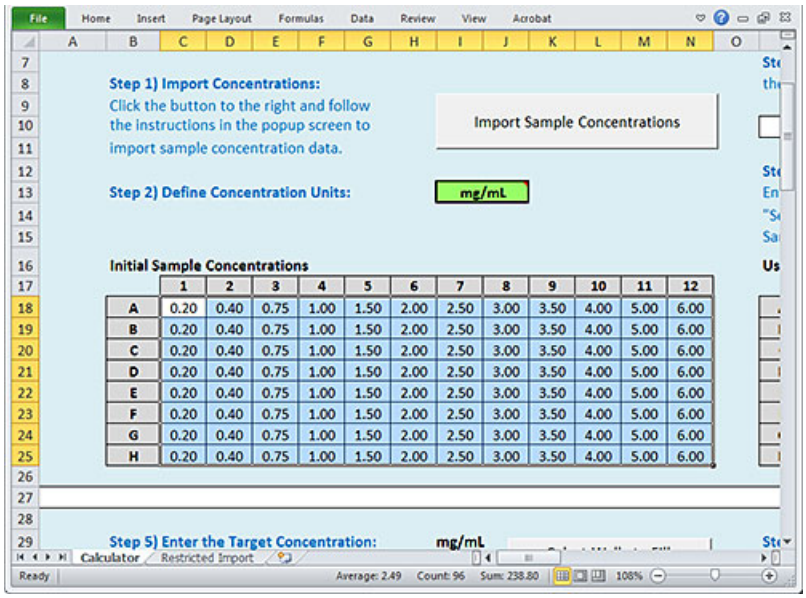
The selected Excel file opens and the Select Data dialog box opens.

5 In the Excel file, select the 8-row by 12-column array of values to be imported, and then click **OK** in the **Select Data** dialog box.

Note: When selecting data from an Excel sheet, the selected range must be an 8-row by 12-column array of values that correspond to the well positions of the samples.



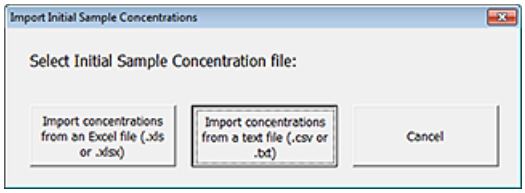
The Excel file closes and the imported values appear in the Initial Sample Concentrations area.



Importing values from a text file

To import the values from a text file (.csv or .txt):

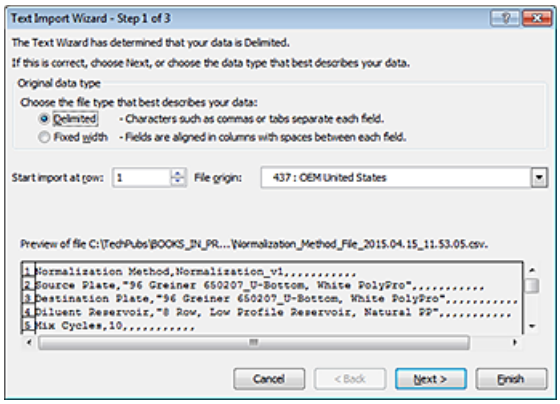
1 Click **Import Sample Concentrations**. The Import Initial Sample Concentrations dialog box opens.



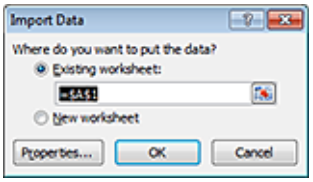
2 Click **Import concentrations from a text file (.csv or .txt)**.

3 In the **Import Text File** dialog box, select the file and click **Import**.

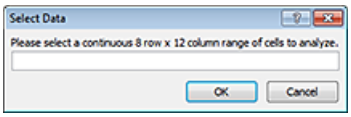
4 In the **Excel Text Import Wizard** that appears, follow the instructions to specify the type of delimited file.



5 In the **Import Data** dialog box, select **Existing worksheet**, and then click **OK**.

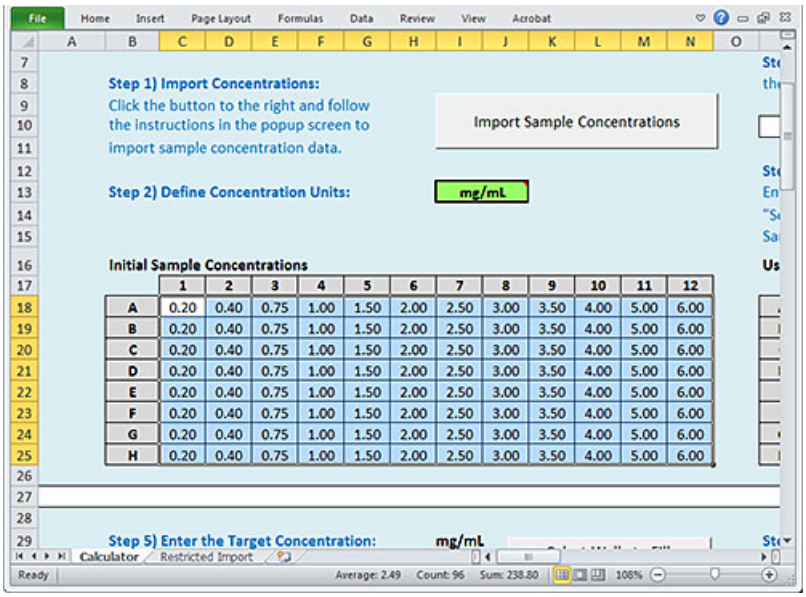


The content of the imported file appears in the Restricted Import worksheet of the Method Setup Tool, and the Select Data dialog box opens.



6 In the imported worksheet, select the 8-by-12 array of values, and then click **OK** in the **Select Data** dialog box.

The selected values appear in the Initial Sample Concentrations area.



Step 2) Define Concentration Units

To define the concentration units:

In the green box next to **Step 2) Define Concentration Units**, verify that the unit of measure for the sample concentration is correct. If necessary, type the correct units.

This input is only a reminder. It has no impact on the subsequent data or volume calculations.

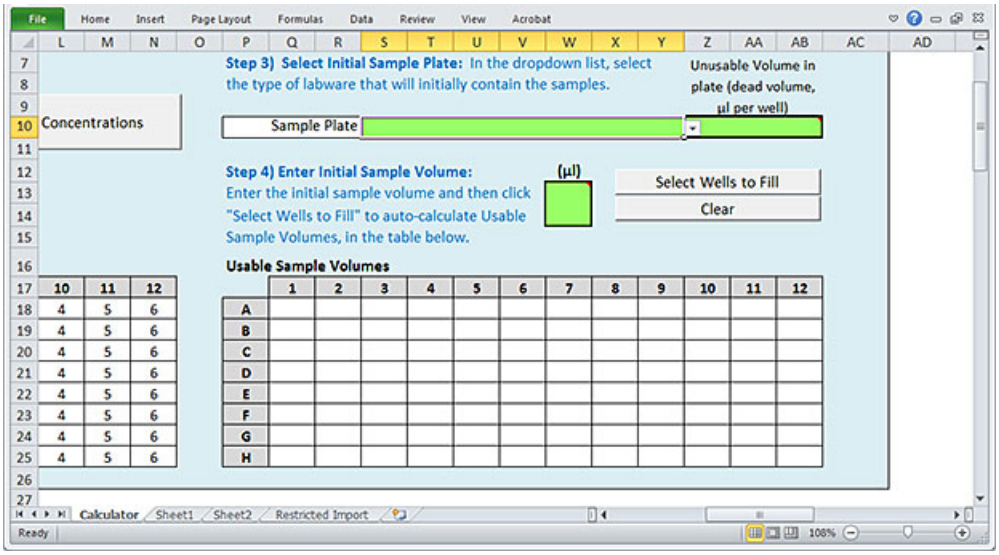
IMPORTANT

Ensure this unit of measure is constant throughout the setup process. The tool does not adjust calculations to accommodate the use of different units for the initial and target concentrations.

Step 3) Select Initial Sample Plate

To select the initial Sample plate:

1 Click the **Sample Plate** box, and then select the labware from the list.



2 Notice the value that automatically appears in the **Unusable Volume** box. To calculate the usable volume, the calculator will subtract this value from the value in Step 4) Initial Sample Volume.

The unusable volume (also known as *dead* volume) is the volume that cannot be reliably aspirated from the microplate well due to pipetting limitations that arise from differences in well-bottom geometry.

The default value for **Unusable Volume** is a recommended setting that is based on extensive testing with the labware. If necessary, you may change the value, for example if the value is not appropriate for the sample liquid.

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

For a list of recommended values for each allowable labware type, see "Labware" on page 588.

Note: The *Labware Reference Guide* also presents labware-specific pipetting overage and maximum well capacity. You can find this guide in the Literature Library page of the Protein Sample Prep Workbench.

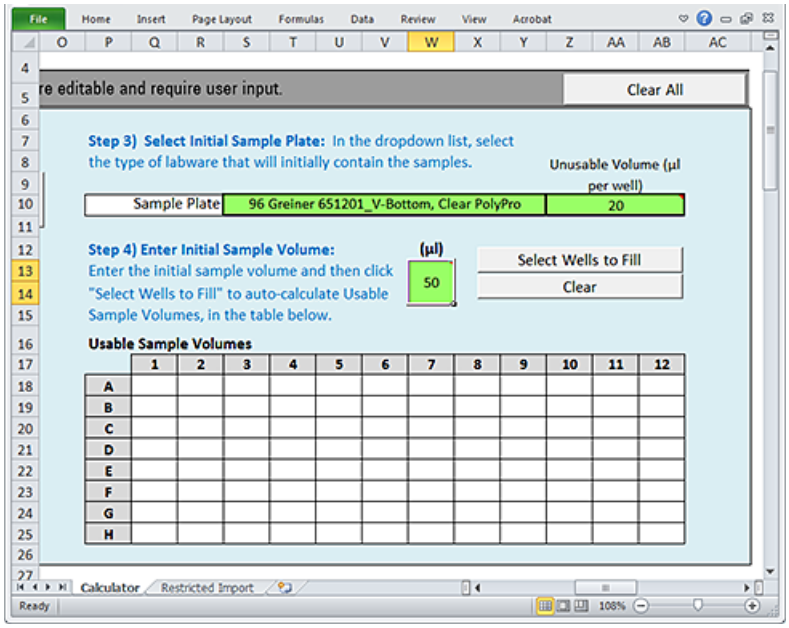
Step 4) Enter Initial Sample Volume

Use the following procedure to define a volume of sample that is initially present in each well of the Sample plate.

To enter the initial Sample volume:

1 In the green **(µL)** box, type the initial sample volume.

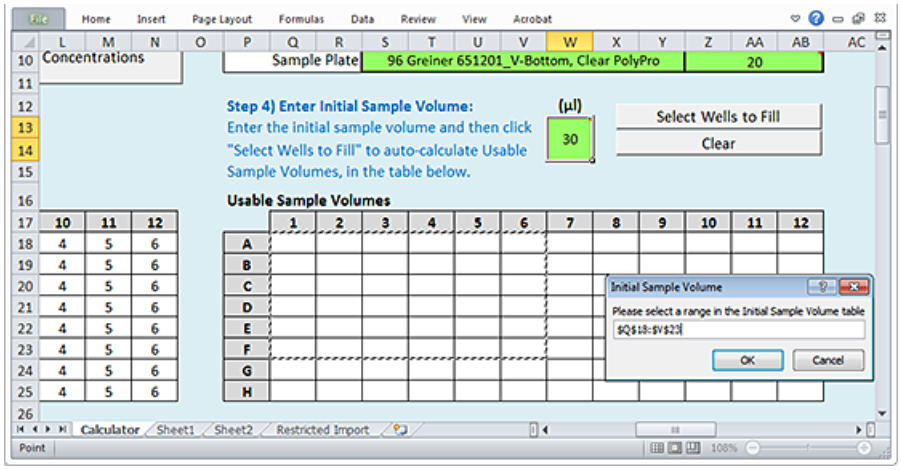
2 Click **Select Wells to Fill**.



3 When the **Initial Sample Volume** dialog box opens:

a Select the array of wells to be filled in the **Usable Sample Volumes** plate grid of the worksheet.

b Click **OK** in the **Initial Sample Volume** dialog box to populate the selected wells of the plate grid.



Note: The values that populate the plate grid represent the volume of the Initial Sample Volume that is usable. This value is the difference between the value you entered in the green box for Initial Sample Volume and the value in the Step 3) Unusable Volume box.

If you want to clear all values from the Usable Sample Volumes plate grid, click Clear.

4 Repeat step 1 to step 3 until you have specified the volume for all the wells in the Sample plate that contain a sample.

Step 5) Enter the Target Concentrations

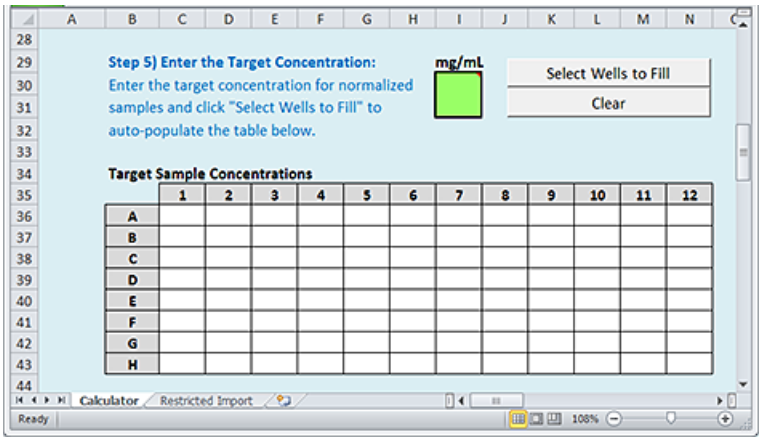
IMPORTANT

Make sure you consider what is possible given the Initial Sample Concentrations and Usable Sample Volumes from Step 1) and Step 4), respectively. Although you may enter values that are not possible or practical given the Initial Sample Concentration and Usable Sample Volume, the tool will flag such values as exceptions when calculating the normalization volumes. You will specify how to handle each exception in a subsequent step.

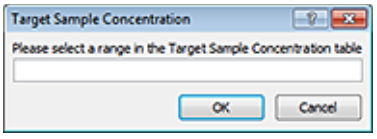
Use the following procedure to specify the desired concentrations after the samples are normalized.

To enter the target concentrations:

1. In the green box, type the concentration.
2. Click **Select Wells to Fill**.



3. When the **Target Sample Concentration** dialog box opens:



   a. Select the array of wells to be filled in the **Target Sample Concentrations** plate grid of the worksheet.

   b. Click **OK** in the **Target Sample Concentration** dialog box to populate the selected wells of the plate grid.

   Note: If you want to clear all values from the Target Sample Concentrations plate grid, click Clear.

4. Repeat step 1 to step 3 until you have specified the concentrations for all the wells.

Step 6) Enter the Target Volume

IMPORTANT

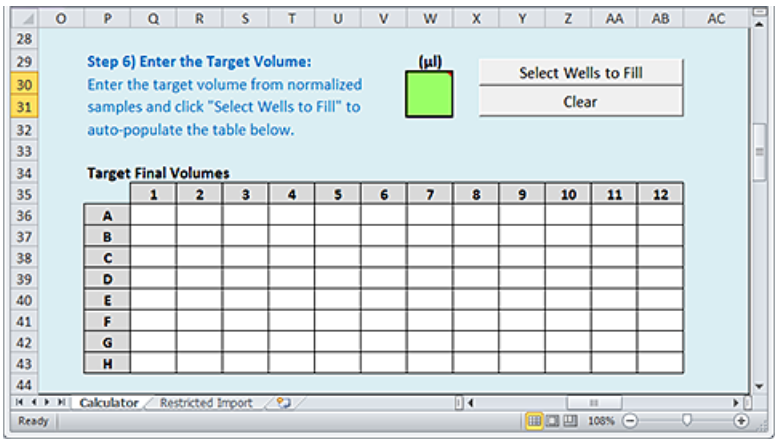
Make sure you consider what is possible given the previous entries. Although you may enter values that are not possible or practical given the Initial Sample Concentration, Usable Sample Volume, or Target Concentration, the tool will flag such values as exceptions when calculating the normalization volumes. You will specify how to handle each exception in a subsequent step.

Use the following procedure to specify the desired volumes after the samples are normalized.

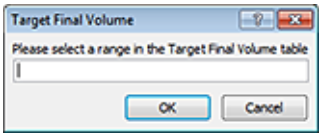
To enter the target volume:

1 In the green **(µL)** box, type a value that you want to apply to multiple wells of the microplate.

2 Click **Select Wells to Fill**.



3 When the **Target Final Volume** dialog box appears:



a Select the array of wells to be filled in the **Target Final Volumes** plate grid of the worksheet.

b Click **OK** in the **Target Final Volume** dialog box to populate the selected wells in the plate grid.

Note: If you want to clear all values from the Target Final Volumes plate grid, click Clear.

4 Repeat step 1 to step 3 until you have specified the volumes for all the wells.

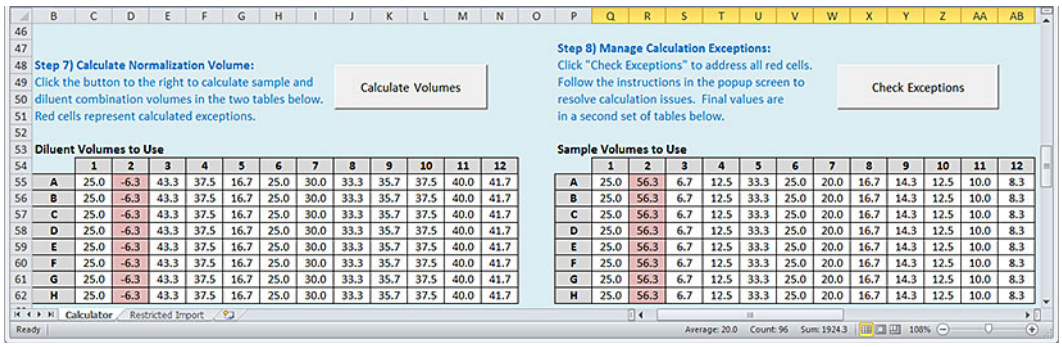
Note: If the next step in the workflow involves an AssayMAP application, all the samples should be at the same target volume.

Step 7) Calculate Normalization Volumes

To calculate the normalization volumes:

Click **Calculate Volumes**. An algorithm uses the information provided in the previous steps to calculate the sample and diluent volumes required to achieve the specified normalization targets. The calculated volumes (µL) are displayed in the following areas:

- Diluent Volumes to Use plate grid
- Sample Volumes to Use plate grid



Any wells with red highlights are considered exceptions, for which the calculated sample or diluent volume values cannot be achieved because the volumes required are either larger than the volumes available in the Sample plate or smaller than practical for the AssayMAP Bravo Platform to transfer with high accuracy.

Step 8) Manage Calculation Exceptions

IMPORTANT

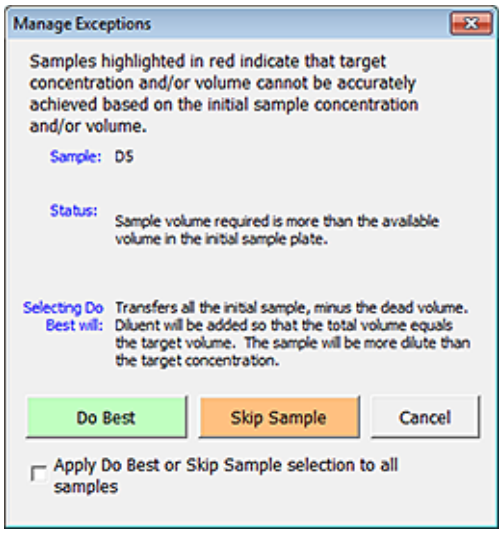
You must click **Check Exceptions** in order to proceed, even if there are no red highlighted wells in the plate grid. The setup tool also performs other important conformity checks at this time.

In this step, you decide how to address each of the exceptions that are indicated by red highlighted wells in the plate grid.

To manage the exceptions:

1 Click **Check Exceptions**. The Manage Exceptions dialog box opens. The descriptions change in the Status and the Selecting Do Best Will areas depending on the conditions that caused the exception for the selected sample. The following figure shows an example.

Figure Example of the Manage Exceptions dialog box



2 Select the option that is appropriate for your samples. The options are as follows:

Option	Description
Do Best	The resulting actions for using this option depend on the conditions that caused the exception. An explanation of the action for the selected sample is displayed in the Selecting Do Best Will area. For descriptions by the type of exception, see the following section, Do Best option and types of exceptions. In general, Do Best adjusts the calculated sample and diluent volumes to get as close to the target concentration as possible while maintaining the target volume. The tool gives a higher priority to the target volume than the target concentration, assuming that the Normalized plate will be used in another AssayMAP Bravo run, and the well volume differences across the plate can lead to unpredictable assay results. The values from the calculations appear in the Expected Concentrations after Normalization plate grid at the bottom of the worksheet. You may apply Do Best in the following ways: • Click **Do Best** for each exception, sample by sample. • Select **Apply Do Best or Skip Sample selection to all samples**, and then click **Do Best**. The action is performed on all the remaining exceptions. After addressing all the exceptions, click **Close** to close the dialog box.

Option	Description
Skip Sample	This option skips the normalization process for the selected sample. If selected, nothing will be transferred into the Normalized plate at the specified well location. You may apply Skip Sample in the following ways: • Click **Skip Sample** for each exception, sample by sample. • Select **Apply Do Best or Skip Sample selection to all samples**, and then click **Skip Sample**. The action is performed on all the remaining exceptions. After addressing all the exceptions, click **Close** to close the dialog box. *Note:* No pipette tip is used for blank wells or skipped wells.
Cancel	Click Cancel to exit the Manage Exceptions dialog box if: • No exceptions remain and you want to move on to the final steps of defining the normalization method. • You want to return to an earlier step in the method setup process to make changes.
Apply Do Best or Skip Sample selection to all samples	The option to perform the Do Best or Skip Sample action on all the remaining wells. Select the **Apply Do Best or Skip Sample selection to all samples** check box, and then click **Do Best** or **Skip Sample**, as applicable. After addressing all the exceptions, click **Close** to close the dialog box.

Do Best option and types of exceptions

If you select the Do Best option, the resulting Bravo actions vary depending on the conditions that caused the exception. The following four conditions cause exceptions:

1. The initial concentration value for a sample is lower than the target concentration for that sample.
2. The usable sample volume value is insufficient to cover the volume required to meet the normalization target concentration or target volume.
3. The calculated sample volume is less than 5 µL, which is the minimum practical volume at which high accuracy and precision (5% CV, 10% Relative Inaccuracy) can be achieved when using the AssayMAP Bravo Platform.
4. The initial concentration for a sample is lower than the target concentration for that sample, and the usable volume in the initial Sample plate is not sufficient to allow for the entire sample to be transferred directly into the Normalized plate.

The following table describes the status messages and Do Best behaviors for each of the four exceptions.

Exception	Status message	Do Best behavior
1	Initial concentration is below the target concentration.	No dilution will take place. Only sample will be transferred to the Normalized plate, which will cause the sample to have a concentration that is lower than the specified target concentration.
2	Sample volume required is more than is available in the Initial Sample plate.	The deficit in sample volume will be substituted with diluent, causing the concentration to be lower than expected, but ensure that the target volume is still met.
3	Sample volume required is less than 5 µL. Five microliters is the minimum volume that can be transferred with a high degree of accuracy.	The AssayMAP Bravo Platform will attempt to perform the normalization as defined, by pipetting less than 5 µL of sample.
4	Initial concentration is below the target concentration and the initial volume is less than the target volume.	The deficit in sample volume will be substituted with diluent, causing the concentration to be lower than expected, but ensure that the target volume is still met.

As the exceptions are managed:

- If Do Best was selected, the new values calculated for the diluent and sample volume are highlighted in the Final Diluent Volume and Final Sample Volume plate grids.
- If Skip Sample was selected, the word “Skip” appears as the value for both the Final Diluent Volume and the Final Sample Volume.

IMPORTANT

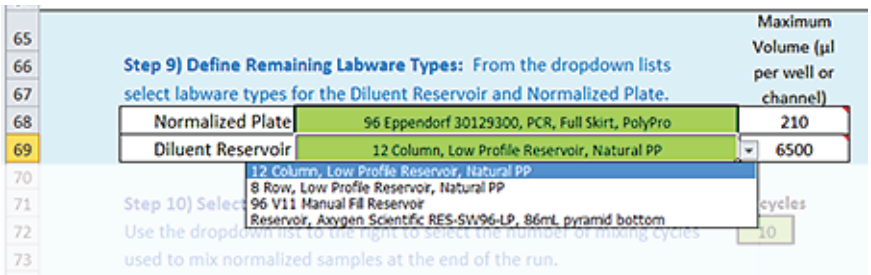
If you have exceptions, make sure you review the **Expected Concentrations after Normalization** values at the bottom of the worksheet before you create the method file.

Step 9) Define Remaining Labware Types

To specify labware for the remaining deck locations:

1 Click the **Normalized Plate** green box, and then select the labware from the list.

Note: This plate will be placed on a Bravo Plate Riser at deck location 6, which is where the final normalized samples will be at the end of the run.



2 Notice the value that automatically displays in the **Maximum Volume (µL per well or channel)** box.

IMPORTANT

If this value has a red highlight, the selected labware cannot accommodate the volume specified in Step 6) Target Volume.

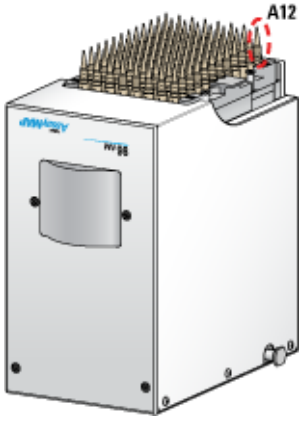
3 Click the **Diluent Reservoir** green box, and then select the labware from the list.

Note: This reservoir will be placed at deck location 4 without a Bravo Plate Riser. The reservoir will supply diluent for the normalization process.

4 Notice the corresponding value in the **Maximum Volume (µL per well or channel)** box. This value minus the dead volume associated with the selected plate type is the volume that the Bravo 96AM Head can access in the selected labware. The Bravo 96AM Head uses a single pipette tip mounted on probe A12 to aspirate the volume.

For example, in a 12-column reservoir, the accessible volume is only the rightmost column, and in an 8-row reservoir, the accessible volume is only the topmost row.

Figure Upside down view of AssayMAP head showing probe A12



Step 10) Select the Number of Mix Cycles

In this step you select the number of mix cycles to perform after all the samples and diluent are added in the Normalized plate. The Bravo 96AM Head presses on the full selection of used pipette tips from deck location 3, and then mixes all the wells at the same time.

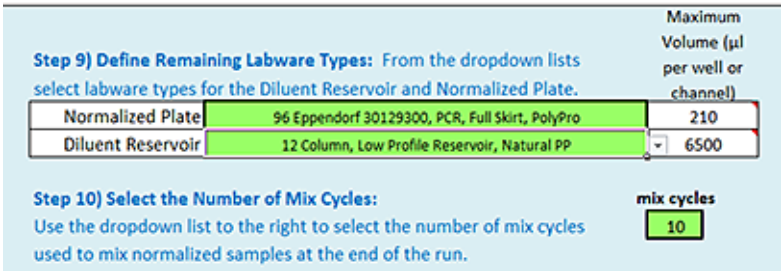
The mixing volume is 75% of the cumulative plate volume, up to the maximum capacity of the pipetting method, where

Capacity for 250 µL pipette tips = 140 µL

Normalization methods for large volumes may require a greater number of mix cycles, which you should determine empirically.

To select the number of mix cycles:

Click the **mix cycles** box, and then select the value from the list.



Step 11) Input Evaporation Correction Factor

In general, a Normalization protocol run of 96 samples on the AssayMAP Bravo Platform requires approximately 110 minutes. During this time, the samples can evaporate, especially by the time the 96th sample is transferred. The optional Evaporation Correction Factor helps to compensate for the sample evaporation.

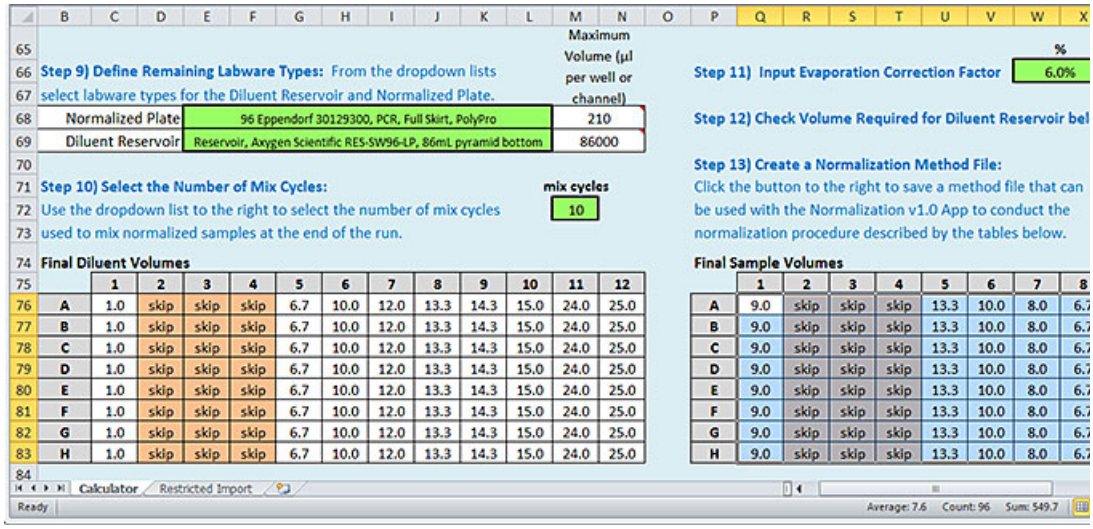
The evaporation rate will vary depending on the sample solution. You should empirically determine the correction factor using test samples. See Assay development guidelines for a detailed explanation of when to use this factor and how to calculate an appropriate value to use.

To account for sample evaporation:

1 In the Step 11) green box, type a value (%) for the correction factor, and then click **Apply Correction Factor**.

The tool automatically adjusts the values in the **Final Diluent Volumes** and **Final Sample Volumes** plate grids. The button label changes to Undo Correction Factor.

Note: If you want to undo the change, click Undo Correction Factor.



Step 12) Check Volume Required for Diluent Reservoir below and Adjust If Necessary

CAUTION **A small volume excess is required in all labware types to ensure proper volume transfer.**

To verify that the Diluent reservoir has sufficient volume:

1 Review the **Diluent required for Diluent Reservoir** area at the bottom of the worksheet. The Method Setup Tool calculates the total fill volume for the Diluent reservoir based on the calculated Final Diluent Volumes and the recommended excess volume for the selected labware type.

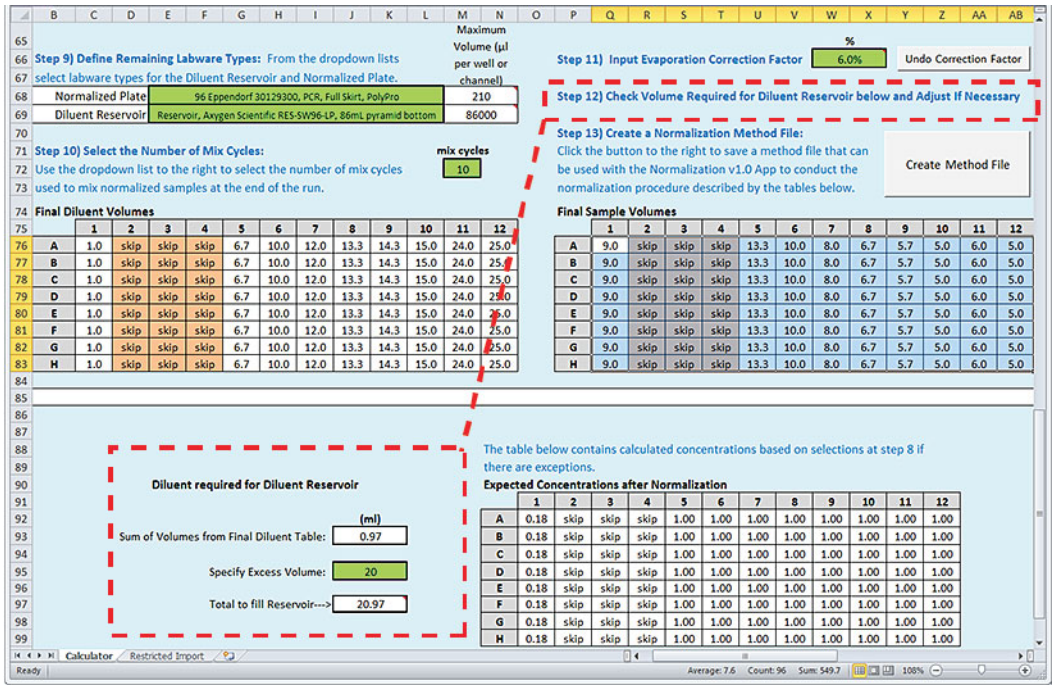
Note: For a list of recommended values for each allowable labware type, see "Labware" on page 588.

2 If a red highlight appears on the **Total to fill Reservoir** value, the diluent volume required is more than the selected labware can hold. In this case, select a different labware option for the **Diluent Reservoir** in Step 9).

Note: If you are using a 12-column reservoir or an 8-row reservoir, the **Total to fill Reservoir** value represents the volume in the rightmost column or topmost row, respectively.

3 Make a note of the **Total to fill Reservoir** volume. You will use this value to prepare the diluent reservoir for the normalization run.

Step 13) Create a Normalization Method File

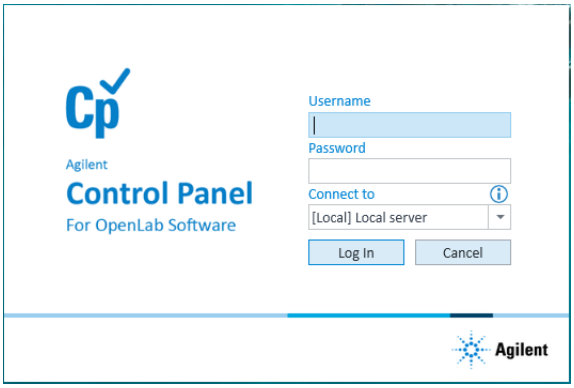


Note: If you manually type the values into the plate grids of the Normalization Method Setup Tool, you may have unexpected results.

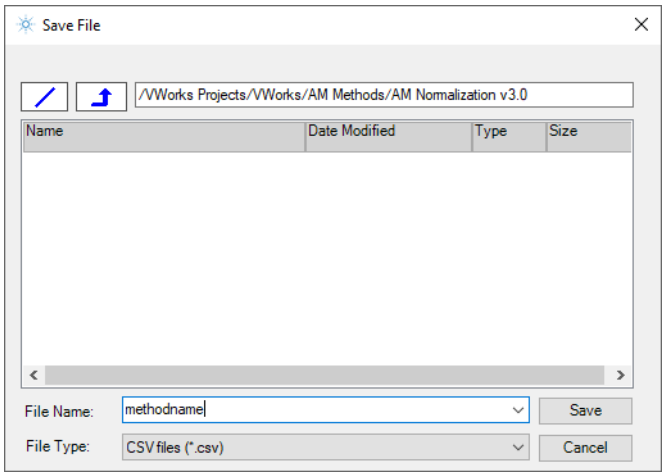
To create the Normalization method file:

1 Click Create Method File.

2 In the login window, type your VWorks user name and password, and click **Log In**.



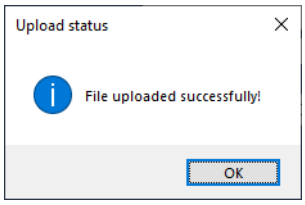
3 In the **Save File** dialog box, specify the file name and the storage location, and then click **Save**.



- VWorks Plus default storage location:
  … /VWorks Projects/VWorks/AM Methods/AM Normalization v3.0
- VWorks Standard default storage location:
  C:\OLSS Projects\VWorks Projects\VWorks\AM Methods\AM Normalization v3.0

Note: The method file is stored as a record of interest (ROI) in an archive (.csv.roiZip). VWorks cannot load files (.roiZip extension) that have been modified or renamed outside of the Protein Sample Prep Workbench or VWorks software.

4 Click **OK** when the uploaded successfully message appears. The Method Setup Tool closes.



Next steps:

If you want to view the method in Microsoft Excel, see "Exporting and importing AssayMAP methods" on page 17

If you are ready to prepare the serial dilution plates, see "Preparing the diluent and samples" on page 609.

Preparing the diluent and samples

Before you start

If you have not already done so, display the method's labware selections and diluent and sample preparation instructions by doing one of the following:

- Open the Normalization utility and select the method. For details, see "Setting up the protocol" on page 611.
- Export the method and view it in Microsoft Excel. For details, see "Exporting and importing AssayMAP methods" on page 17.

IMPORTANT To minimize evaporation, fill the Sample and Diluent plates immediately before run time or keep them covered until you run the protocol.

CAUTION **Incorrect labware selections can cause a hardware collision, resulting in equipment damage. Ensure that the selections in the method exactly match the physical labware present on the Bravo deck.**

Preparing the Diluent reservoir

To prepare the Diluent reservoir:

Prepare the Diluent plate by putting the volume calculated by the Normalization Method Setup Tool in position A12. All the diluent will be aspirated from this location.

Note: If you are using a 12-column reservoir or an 8-row reservoir, the Total to fill Reservoir value represents the volume in the rightmost column or topmost row, respectively.

Transferring samples to the microplate

To transfer the samples to the microplate:

1. Ensure that the labware type for the initial samples plate matches the labware specified in the Normalization Method Setup Tool.
2. Run the Reagent Transfer utility or Reformatting utility to transfer the samples. For instructions, see one of the following:
   - "Reagent Transfer v3.0 User Guide" on page 525
   - "Reformatting v3.0 User Guide" on page 623

   Ensure that the volume and well positions are as specified in the Normalization Method Setup Tool.
3. If necessary, centrifuge the sample labware to remove bubbles.

Running the protocol

The Normalization protocol does the following:

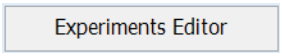
- Washes and dries the syringes.
- Transfers diluent and sample to the normalized plate.
- Mixes all the samples in the normalized plate.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the Normalization Setup Method. See "Setting up a Normalization method" on page 590.
- Display the method's labware selections and diluent and sample preparation instructions by doing one of the following:
  - Open the Normalization utility and select the method. For details, see "Setting up the protocol" on page 611.

  - Export the method and view it in Microsoft Excel. For details, see "Exporting and importing AssayMAP methods" on page 17.
- Prepare the diluent and samples. See "Preparing the diluent and samples" on page 609.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- Run the Startup protocol to prepare the AssayMAP Bravo Platform for the run. See the System Startup/Shutdown v3.0 User Guide utility.

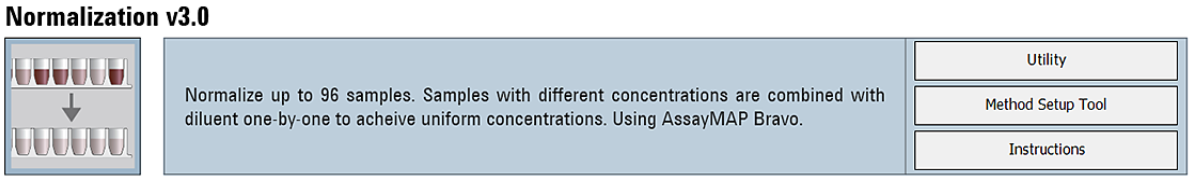
Setting up the protocol

Before starting the protocol, make sure the appropriate selections and values are specified in the Normalization utility.

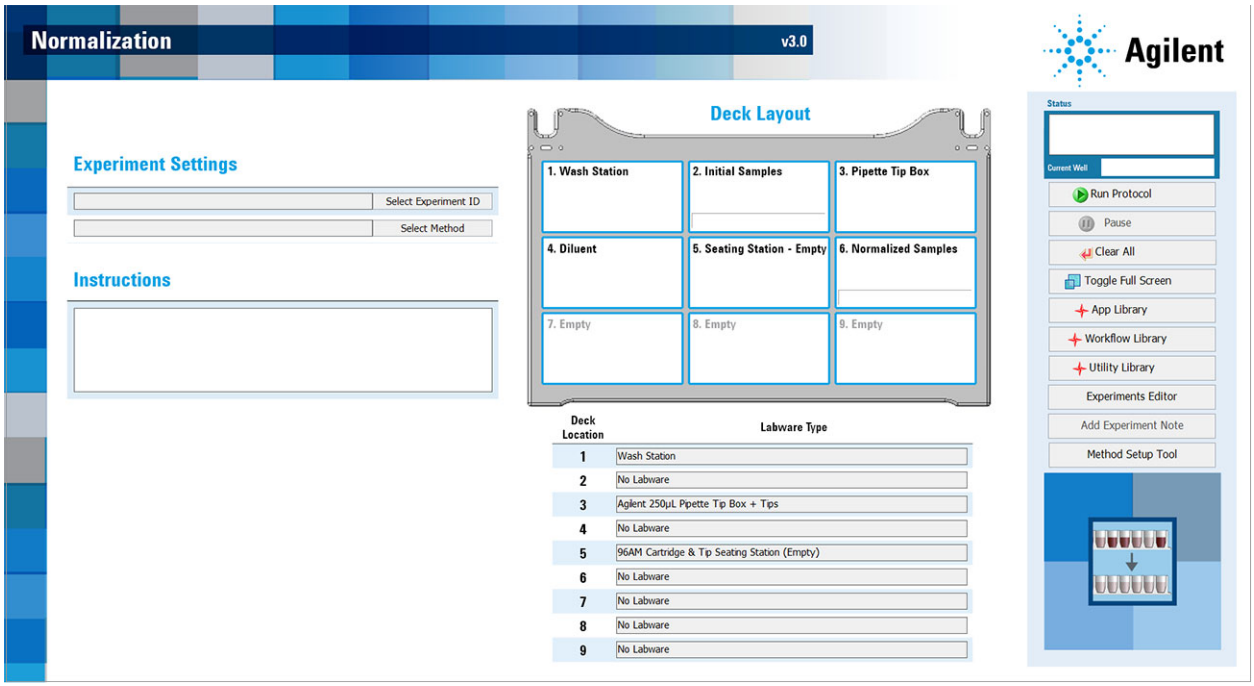
To set up the protocol:

1 Open the **Utility Library**.

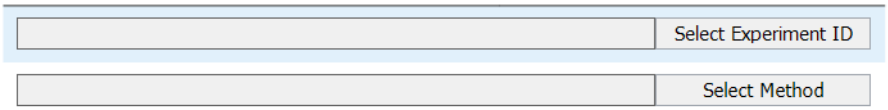
2 Locate **Normalization**, and then click **Utility**.



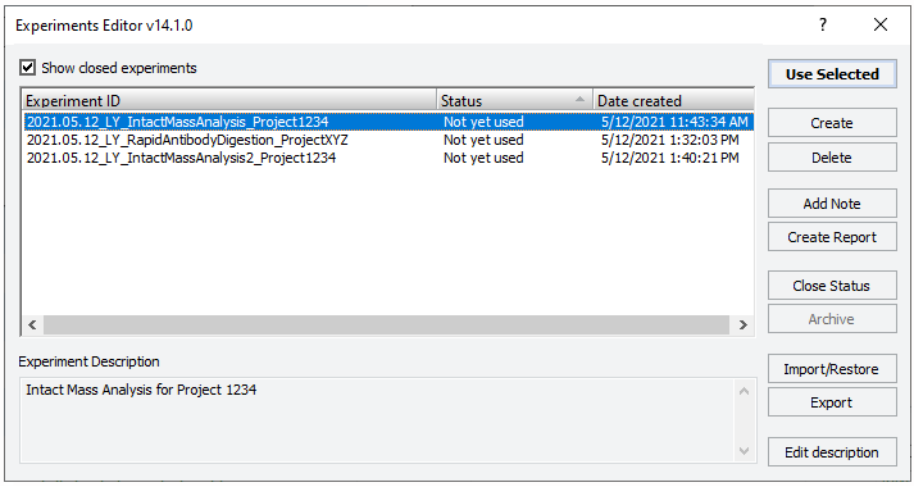
The Normalization utility opens.



3 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



4 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

5 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

The form displays the method labware selections and diluent preparation instructions.

- To run the selected method, go to "Starting the protocol run" on page 613.
- To create or modify a method, see "Setting up a Normalization method" on page 590.

  VWorks Plus. Administrator or technician privileges are required to create and modify methods.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. You must use pipette tips for the mock run.

Starting the protocol run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

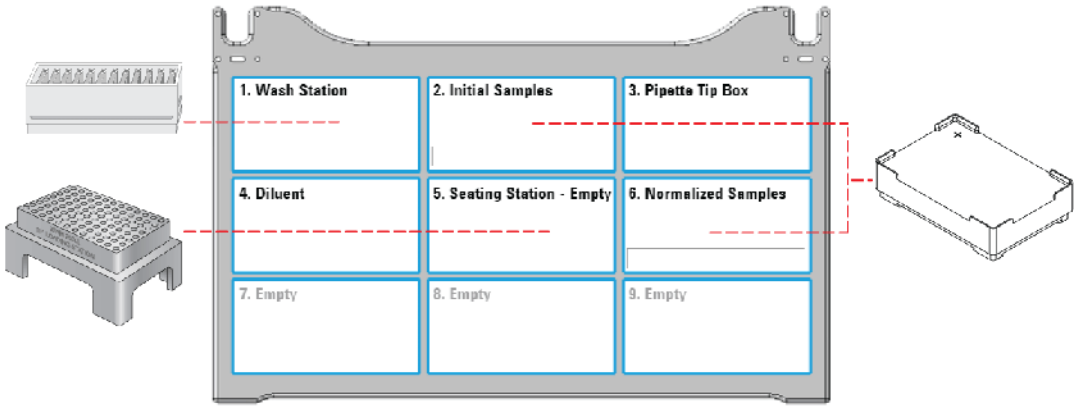
To start the protocol run:

1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

- The Bravo Plate Risers are securely in place at deck locations 2 and 6. The initial samples plate is seated on the Bravo Plate Riser at deck location 2. The normalized samples plate is seated on the Bravo Plate Riser at deck location 6.
- The Diluent reservoir is at deck location 4.
- The tip box full of fresh pipette tips is at deck location 3 and the empty 96AM Cartridge & Tip Seating Station is at deck location 5.

  Note: A full tip box is required for each run even if the run uses less than 96 pipette tips.

  Note: The protocol will transfer the pipette tips to the seating station.



CAUTION **To prevent a potential collision, ensure that no thermal plate insert is on the Peltier Thermal Station installed at deck location 4.**

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.

Note: After the protocol run starts, you can walk away from the AssayMAP Bravo Platform for the duration of the protocol. In general, 96 samples requires approximately 110 minutes.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

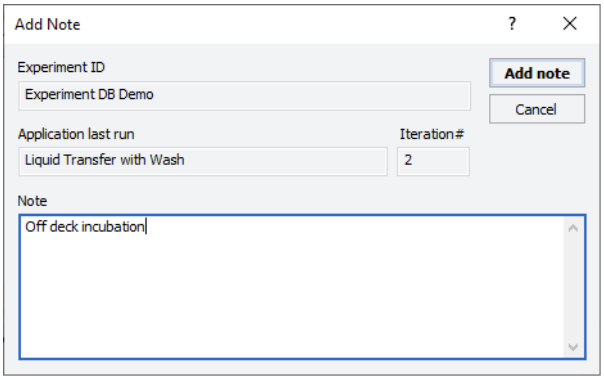
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

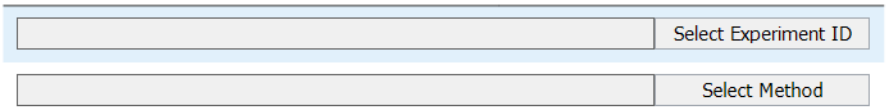
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional.* Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines

This topic provides guidelines for adjusting the protocol, and notes about the utility.

Note: See the *Labware Reference Guide* for labware-specific maximum well capacity and other details. You can find this guide in the Literature Library page of the Protein Sample Prep Workbench.

Correcting for loss due to evaporation

Evaporation can cause problems for any normalization process, whether conducted manually or using automation. The time difference between the normalization of the first sample (A1) and the last sample (H12), leads to progressively increasing atmospheric exposure across the samples. This causes a gradual increase in the mass of sample that is transferred into the normalized sample plate, which leads to higher than expected concentrations for samples with long exposure times. The following figure shows the order in which the Normalization utility normalizes the samples. The

utility starts with column 1 on the left and finishes with column 12 on the right. Without any evaporation correction, you would expect to see the normalized concentrations increase across the plate from left to right.

Figure Run order: the sequence in which samples are normalized

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Step 11 of the Normalization Method Setup Tool contains an optional Evaporation Correction Factor to correct for this problem. The tool applies the factor to the calculated normalization volumes. The factor progressively decreases the volume of Initial Sample (deck location 2) that is used to prepare each normalized sample (deck location 6). This reduction in sample volume is then substituted with diluent to maintain the same target volume.

The Evaporation Correction Factor represents the relative increase in concentration across an entire plate of 96 samples. For example, if the target concentration is 1.00 mg/mL, and after normalization A1 = 1.00 mg/mL and H12 = 1.05 mg/mL, then the relative increase in concentration across the plate would be 0.05 or 5%. Thus, the recommended Evaporation Correction Factor would be 5%.

How to determine an appropriate Evaporation Correction Factor

To determine an appropriate percentage to use for the Evaporation Correction Factor, run a representative normalization protocol to determine the amount of concentration increase observed. The representative normalization run can use an inexpensive reagent, such as bovine serum albumin (BSA). Example 1 describes how to determine the Evaporation Correction Factor from a full plate of normalized results.

Example 1. Determining the Evaporation Correction Factor from a full plate of normalization results.

- Target Concentration = 1.0 mg/mL
- Normalization Run Results:

Figure Example 1 normalized sample concentration (mg/mL) without an Evaporation Correction Factor

		Column											
		1	2	3	4	5	6	7	8	9	10	11	12
Row	A	1.00	1.01	1.01	1.02	1.02	1.03	1.03	1.04	1.04	1.05	1.06	1.07
	B	0.98	0.99	1.00	1.00	1.01	1.01	1.02	1.02	1.03	1.03	1.04	1.05
	C	0.98	0.99	1.00	1.01	1.01	1.01	1.02	1.02	1.03	1.03	1.04	1.05
	D	0.99	0.99	1.00	1.00	1.01	1.02	1.02	1.02	1.03	1.04	1.04	1.05
	E	0.99	1.00	1.00	1.01	1.01	1.02	1.02	1.02	1.03	1.05	1.04	1.05
	F	1.00	1.01	1.02	1.02	1.02	1.03	1.03	1.03	1.04	1.04	1.06	1.06
	G	1.00	1.01	1.02	1.02	1.03	1.04	1.04	1.04	1.06	1.06	1.11	1.06
	H	0.98	1.00	1.00	0.99	1.01	1.02	1.02	1.03	1.03	1.04	1.04	1.05

To calculate the Evaporation Correction Factor for Example 1:

1 Use the following equation to calculate relative inaccuracy (RI) for each of the 96 samples with respect to the target concentration:

$$RI_w = \frac{C_w - C_o}{C_o}$$

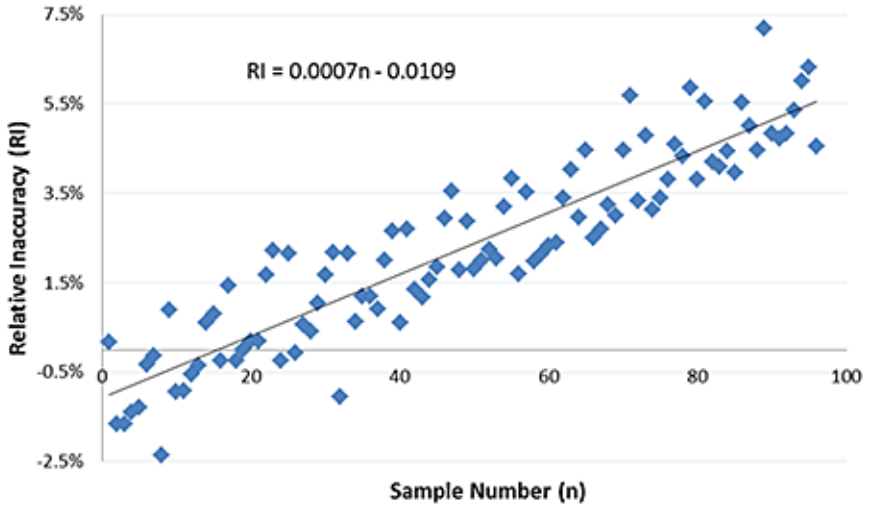
where,

RI_w is the relative inaccuracy for well location *w*

C_w is the concentration for well location *w*

C_o is the target concentration

2 Plot the relative inaccuracy (RI) values against sample number (n), along with a linear regression line.



3 The equation for the regression line is used to solve for RI when n = 96. This gives the relative increase in concentration across the plate, and thus the recommended Evaporation Correction Factor. The calculated factor for this example is as follows:

Evaporation Correction Factor = 0.0007 × 96-0.0109 = 0.056 = 5.6%

The same process can be followed when using a partial plate of normalized samples, as Example 2 demonstrates.

Example 2: Determining the Evaporation Correction Factor from a partial plate of normalization results.

- Target Concentration = 1.0 mg/mL
- Normalization Run Results for Partial Plate:

Figure Example 2 normalized sample concentration (mg/mL) without an Evaporation Correction Factor

		Column											
		1	2	3	4	5	6	7	8	9	10	11	12
Row	A	1.00	1.00	1.00	1.00	1.02	1.02	1.01	1.01				
	B	0.98	0.98	1.01	1.00	1.00	0.99	1.02	1.02				
	C	0.98	1.01	1.01	1.00	1.01	1.02	1.03	1.02				
	D	0.99	0.99	1.00	1.02	1.00	1.01	1.01	1.03				
	E	0.99	0.99	1.01	1.02	1.01	1.01	1.03	1.04				
	F	1.00	0.99	1.00	1.00	1.02	1.01	1.01	1.02				
	G												
	H												

To calculate the Evaporation Correction Factor for Example 2:

1 Calculate the relative inaccuracy (RI) for each of the 48 samples with respect to the target concentration. See Example 1 for the equation to calculate RI.

2 Plot the relative inaccuracy (RI) values against sample number (n), along with a linear regression line. The following figures show how the sample number (n) would change for this partial plate example.

Figure

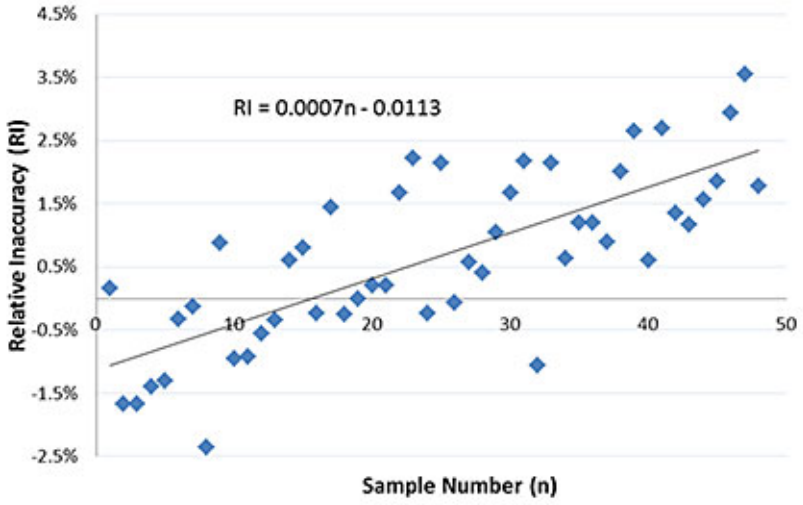


Figure Sample run order: sequence in which the samples were normalized

		Column											
		1	2	3	4	5	6	7	8	9	10	11	12
Row	A	1	7	13	19	25	31	37	43				
	B	2	8	14	20	26	32	38	44				
	C	3	9	15	21	27	33	39	45				
	D	4	10	16	22	28	34	40	46				
	E	5	11	17	23	29	35	41	47				
	F	6	12	18	24	30	36	42	48				
	G												
	H												

3 The equation for the regression line is used to solve for RI when n = 96. This gives the relative increase in concentration across the plate, and thus the recommended Evaporation Correction Factor.

   Evaporation Correction Factor = 0.0007 × 96-0.0113 = 0.056 = 5.6%

Note: Even though only 48 sample were run in this example, the Evaporation Correction Factor is still calculated by solving the regression equation for n = 96. This is because the Evaporation Correction Factor is expressed as the percent increase across an entire plate of 96 samples. Because we don't have a full plate of results, the correction factor must be determined by extrapolation.

Conditions that can affect evaporation

Three factors that are known to affect the evaporation that is observed when using the Normalization utility are:

1 Atmospheric conditions in the laboratory

   The temperature and humidity of a laboratory affect the amount of evaporation that is observed with samples that are exposed to atmosphere. Many laboratories have environmental management systems to help maintain constant atmospheric conditions. These environmental controls will help to reduce changes in the amount of evaporation that is observed, but slight changes in the Evaporation Correction Factor may be required to maintain a consistent normalization results, especially with changing seasons.

2 Sample Plate labware type

   The Initial Sample Plate labware type can have a significant impact on the amount of evaporation that is observed. Different well geometries and volume capacities can impact the rate of evaporation that will be observed.

   - *Well geometry*. Plates with larger diameter wells provide greater surface area for interaction with the open atmosphere, leading to faster evaporation rates.
   - *Volume capacity*. Consider a scenario where Plate 1 has a 100-µL capacity and Plate 2 has a 10-µL capacity. If both plates lose 5 µL of diluent to evaporation, the resulting concentration in Plate 1 would increase by a factor of ~1.05, while the concentration in Plate 2 would increase by a factor of 2.

3 Solvent

   The solvent in which the sample is suspended will dramatically affect the evaporation rate. When determining the evaporation rate, use the same sample solvent that will be used during the normalization run using real samples.

Pipetting accuracy and liquid classes

IMPORTANT

Agilent does not guarantee specific accuracy and precision results for the Normalization utility because accuracy and precision are too dependent on the composition and liquid properties of the samples and diluent used.

The following table lists the default liquid classes that the Normalization utility uses.

Liquid class name	When to use this liquid class
AM_Normalization_diluent_20-150ul	High-volume transfers of diluent (20 to 150 µL)
AM_Normalization_diluent_5-20ul	Low-volume transfers of diluent (5 to 20 µL)
AM_Normalization_sample_20-150ul	High-volume transfers of sample (20 to 150 µL)
AM_Normalization_sample_5-20ul	Low-volume transfers of sample (5 to 20 µL)

These default liquid classes should give acceptable accuracy results for most samples types that will be used with the Normalization utility. Pipetting accuracy and precision can be greatly affected by liquid properties, such as viscosity and surface tension. If a sample or diluent has properties that are different from simple aqueous solutions, then these liquid classes might not give results within the expected accuracy and precision range.

If unacceptable accuracy and precision results are observed, you may modify these liquid classes to achieve acceptable results. See the About Liquid Classes section of the *VWorks Version 4 Automation Control Setup Guide* for more information about liquid classes, and instructions on how to modify them to achieve the specific pipetting characteristics.

Automation movements during the protocol

This section describes the basic automation movements of the AssayMAP Bravo Platform during a Normalization run.

Protocol step	Head moves to deck location...	Action
1. Syringe Wash	1	Performs 1 external syringe wash at the wash station.
2. Syringe Drying	1	Performs 4 syringe aspirate-and-dispense cycles above the wash station to cycle air in and out of the syringes. The syringes move over the chimneys after each cycle to remove any droplets that were pushed out of the syringes during the cycle.
3. Initial Tip Transfer	3	Presses on all the 250-µL pipette tips from the tip box.
	5	Ejects all the pipette tips into the seating station.
4. Single Tip Pickup	5	Uses probe A12 of the head to pick up the next available individual pipette tip.
5. Diluent Transfer	4	Aspirates diluent (well A12) into the pipette tip.
	6	Dispenses the diluent into a specific well in the Normalized plate.
6. Sample Transfer	2	Aspirates sample from the Sample plate into the pipette tip.
	6	Dispenses the sample into the same well in the Normalized plate that was used for the Diluent Transfer process.
7. Single Tip Eject	3	Ejects the used pipette tip into the tip box. *Note:* The tip box location matches the well location of the normalized sample that the pipette tip was used to prepare.
8. Additional Transfers	various	Repeats steps 2 through 5 for every sample in the Sample plate.
9. Used Tip Pickup	3	Presses on all the used pipette tips from the tip box.
10. Mixing	6	Mixes all the samples in the Normalized plate.
11. Final Tip Ejection	3	Ejects the used pipette tips into the tip box.

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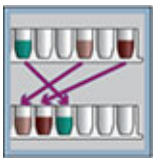
17 Reformatting v3.0 User Guide

This chapter contains the following topics:

Utility description



Reformatting v2.0. This utility automatically transfers sample from any well in a 96-well source plate to any well in a 96-well destination plate. You can aspirate repeatedly from the same source and dispense into multiple destination wells. You can draw from multiple source wells and dispense into a single destination well. For example, you can use the utility to perform random hit picking, to pool samples, or to add an internal standard to selected wells in a Serial Dilution plate.

You use the Reformatting Method Setup Tool to create a method that specifies the following:

- List of samples to be reformatted
- Labware for the source and destination plates
- Volume to be transferred (5–1000 µL)
- Liquid-handling parameters, including options to prewet the pipette tips, specify a blowout volume, choose a liquid class, mix the source and destination wells, and specify a pre-existing volume in the destination wells

The utility uses your saved method to transfer the specified volume of source sample into the designated wells of the destination plate.

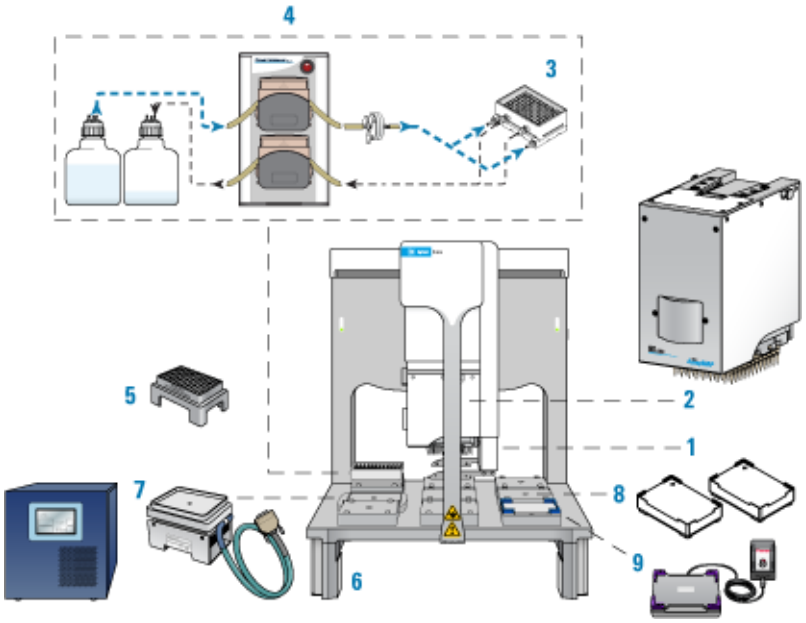
Before you start

This topic lists the required hardware, software, and labware for running the Reformatting utility. If you have questions about these items, contact Agilent Customer Service.

Hardware

The AssayMAP Bravo Platform is required for running the AssayMAP protocols. The following figure and table describe the platform components.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Bravo Risers, 14.6 cm
7	Peltier Thermal Station with STC controller
8	Plate Risers, 2.84 cm (two)
9	Orbital Shaking Station with Control Unit

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

Labware

The Reformatting protocol requires the following labware:

- Full tip box of 96 250-µL pipette tips, Agilent 19477-002

  Note: A full tip box is required for each run even if the run uses less than 96 pipette tips.
- Labware for the source plate and destination plate

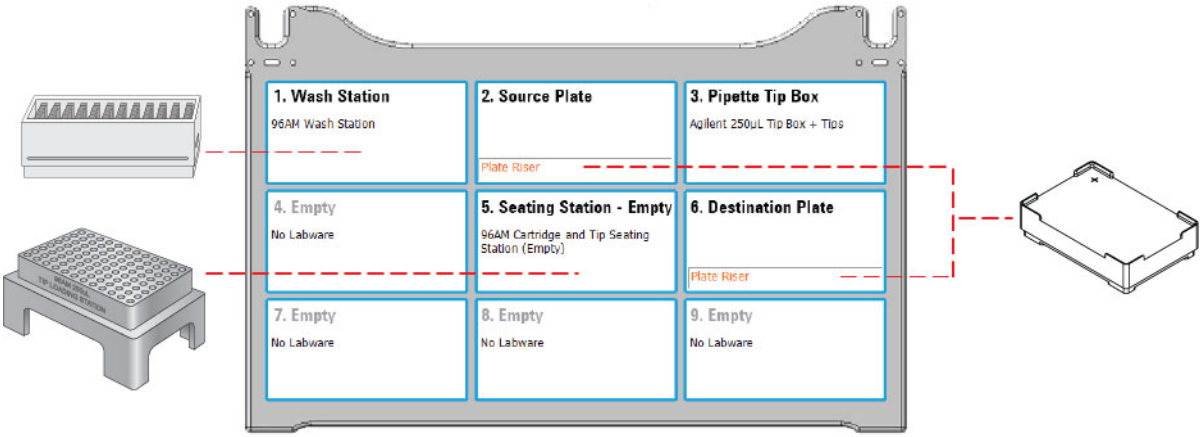
The following tables provide a complete list of labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Using different pipette tips, such as filtered tips, will result in equipment damage. Ensure that you use the standard 250-µL pipette tip (Agilent 19477-002).**

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Accessory and labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
96 ABgene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	2, 6
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	2, 6
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	2, 6
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	2, 6
96 Eppendorf 96-500_V-bottom, Clear PolyPro	Eppendorf 96/500	2, 6
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	2, 6
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	2, 6
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	2, 6
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	2, 6
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	2, 6
96 Thermo Matrix 3732, V-bottom, 0.75ml Storage Tubes	Thermo Fisher Scientific 3732	2, 6
96 Thermo Matrix 3735, V-bottom, 500µl Storage Tubes	Thermo Fisher Scientific 3735	2, 6
96 Thermo Matrix 3744, V-bottom, 500µl ScrewTop Storage Tubes	Thermo Fisher Scientific 3744	2, 6
96 Waters 186005837, Clear PolyPro	Waters 186005837	2, 6
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	2, 6
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	2, 6

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

The unusable volume (also known as *dead* or overage volume) is the volume that cannot be reliably aspirated from the microplate well due to pipetting limitations that arise from differences in well-bottom geometry. The following table provides some general guidelines on how different microplate geometries can affect this value.

Table Guidelines on how microplate geometry can affect volume requirements

Well-bottom geometry	Examples	Unusable volume expectations
Flat bottom	Greiner 675801	The worst plates for minimizing dead volume. Liquids are attracted to the intersection of the plate bottom and side walls.
Round (U) bottom	Greiner 650201, Greiner 650207	Good options for minimizing dead volume. A round bottom forces liquid to collect in the center of the well.
V-bottom and PCR	• V-Bottom: Greiner 651201, Corning Costar 3363 • PCR Plate: Eppendorf 30129300, Greiner 652270	The best plates for minimizing dead volume. Steep side walls force liquid to collect in the center of the well, making it easy to access.

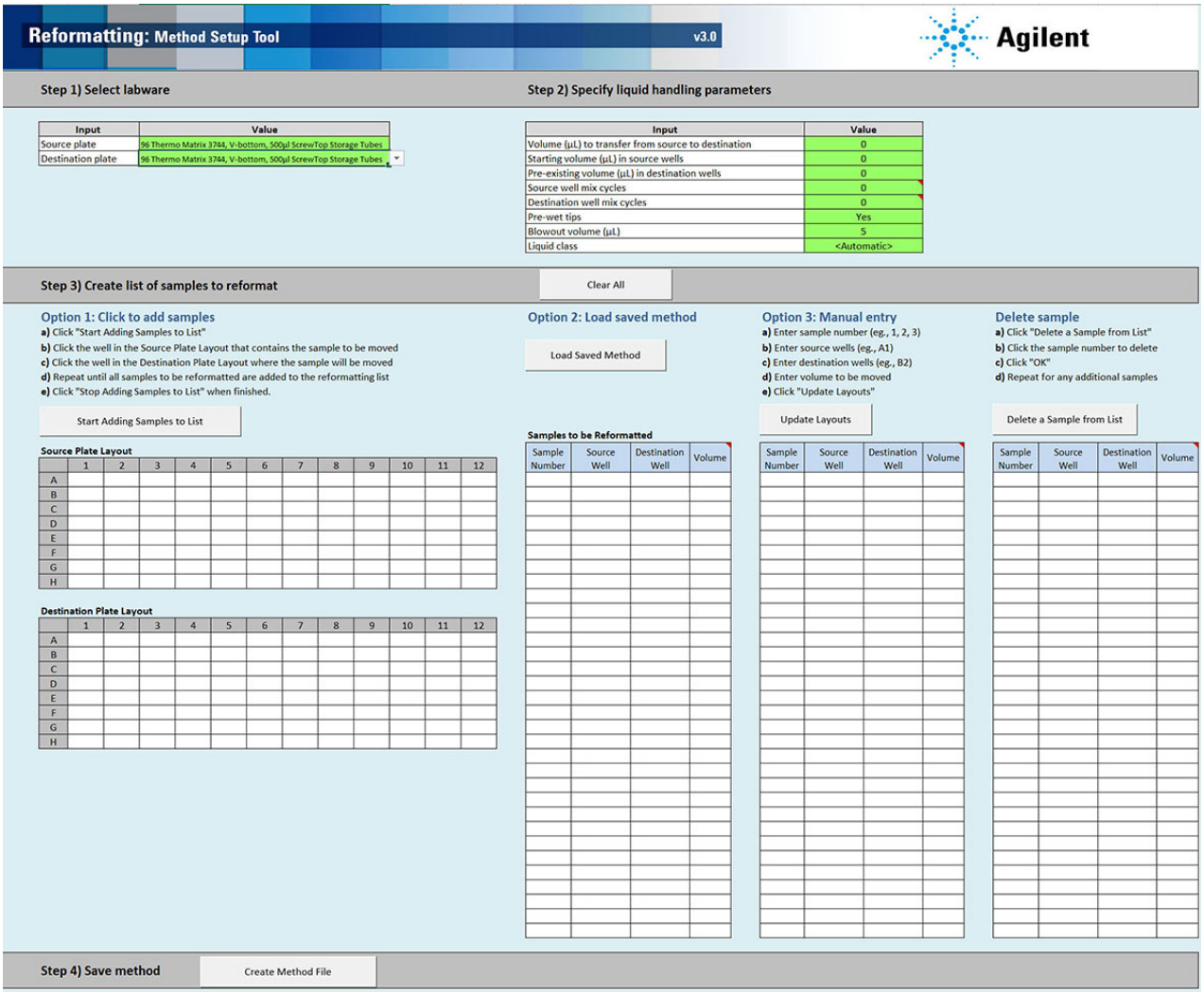
For volume overage recommendations by labware type, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Setting up a Reformatting method

The Method Setup Tool is a Microsoft Excel-based tool that steps you through the process of creating and saving a method file that contains all the information required to run the Reformatting protocol on the AssayMAP Bravo Platform. This tool enables you to define the location of the samples to be transferred, the volumes to be transferred, and the liquid-handling parameters to be used.

Note: When you select a method in the Reformatting utility, the form displays the corresponding labware selections and source and destination plate instructions.

Figure Reformatting Method Setup Tool

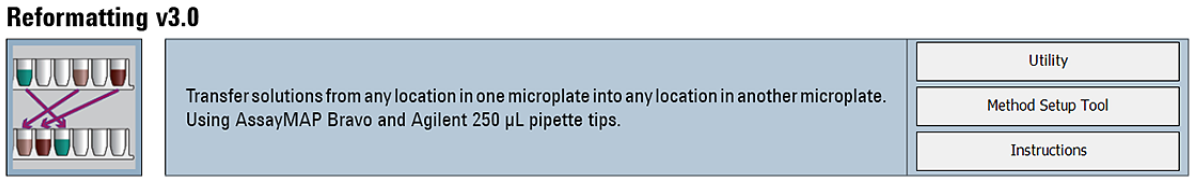


Opening the Method Setup Tool

You can open the tool from the Utility Library or from the Reformatting form.

To open the Method Setup Tool:

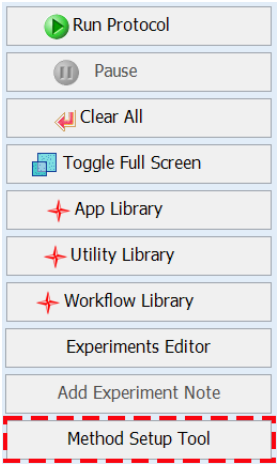
1 In the **Utility Library**, locate the **Reformatting** banner.



2 Click one of the following buttons:

- **Method Setup Tool**. Microsoft Excel starts and displays the Method Setup Tool.
- **Utility**. The Reformatting utility opens.

In the navigation pane, click **Method Setup Tool**. Microsoft Excel starts and displays the Method Setup Tool.



IMPORTANT

In Microsoft Excel, ensure that you enable content.

Overview of steps in Method Setup Tool

The Method Setup Tool has 4 distinct steps. The following table provides an overview of the steps.

Table Overview of steps in Method Setup Tool

Step		Description	
1	Select labware	Click the green **Source plate** box, and then select the labware to be placed on the plate riser at deck location 2. Click the green **Destination plate** box, and then select the labware to be placed on the plate riser at deck location 6 If multiple transfers will be pooled in a single destination well, ensure that the well has sufficient capacity.	
2	Specify liquid handling parameters	Enter values for the following inputs:	Default value (Range)
		Volume (µL) to transfer from source to destination *Note:* <5 µL is possible but the accuracy and precision starts to decrease below 5 µL.	0 (5–1000)
		Starting volume (µL) in source wells	0 (0–1000)
		Pre-existing volume (µL) in destination wells	0 (0–995)
		Source well mix cycles *Note:* The number of mix cycles is dependent on the volume, viscosity, and size of molecules in the solution being mixed.	0 (0–100)

Step		Description	
		Destination well mix cycles If transferring from multiple source wells into a single destination well, additional off-deck mixing may be required because the mixing volume is based on a single transfer volume.	0 (0–100)
		Pre-wet tips	Yes (No, Yes)
		Blowout volume (µL)	5 (0–50)
		Liquid class	Automatic (presets or custom)
3	Create list of samples to reformat	Use one or a combination of the following options to create and edit the list of samples: • Option 1. Select the source and destination well locations for each sample on the plate maps. • Option 2. Load a saved method. *Note:* The saved method must be exported in order to use Option 2. • Option 3. Manually enter the source and destination locations for each sample in the sample table.	
4	Save method	Click **Create Method File**. The setup tool creates a comma-separated value (.csv) text file that you use to run the utility.	

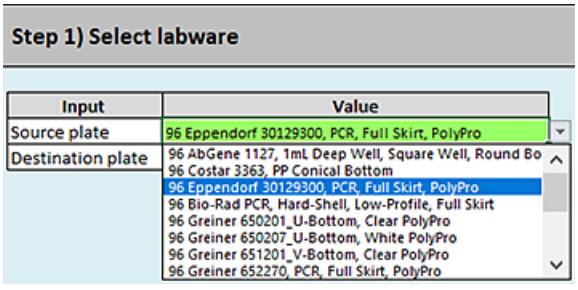
Step 1) Select labware

For the labware options at each deck location, see "Labware" on page 626.

To select the labware:

1 In the cell next to **Source plate**, select the labware that you are placing on the plate riser at deck location 2 as follows:

Click the green cell, and then click the arrow that appears. In the list, click the labware type to be used.



2 In the cell next to **Destination plate**, select the labware that you are placing on the plate riser at deck location 6 as follows:

Click the green cell, and then click the arrow that appears. In the list, click the labware type to be used.

IMPORTANT

If transfers from multiple source wells will be pooled in a single destination well, be sure that the destination well has enough capacity to receive these multiple transfers. The software will not alert you to this type of error.

Step 2) Specify liquid handling parameters

Note: Only the cells highlighted in green are editable.

To specify the liquid handling parameters:

Type or select the values for the following inputs:

Input	Description
Volume (µL) to transfer from source to destination	The volume to be aspirated from the specified well in the source plate and then dispensed in the specified well in the destination plate. *Note:* This value is automatically entered into the list of samples to be reformatted in step 3, option 1 of the Method Setup Tool. You can use step 3, option 3 to edit the volume on a sample-by-sample basis. Default: 0 (µL) Range: 5–1000 (µL) *Note:* Samples <5 µL are possible using step 3, option 3, but samples <5 µL may not have good accuracy or precision.

Input	Description
Starting volume (µL) in source wells	The initial volume in each well of the source plate before the transfer starts. The software uses this volume for error checking and to calculate the liquid height in the wells to facilitate proper dynamic tip behavior. The software error checking assumes that the volume is the same in each well of the plate. **If well volumes vary**, enter the smallest volume as the Starting volume. By doing so, the error checking can alert you if you enter a value to be transferred that is larger than the initial volume. Also, you should enter the value for the smallest source well volume so as not to introduce bubbles while mixing. Note that the larger volume wells may not mix as well as the smaller volume wells because the mixing volume is based on the value on the Starting volume in source wells setting. **If doing multiple transfers from a single source well**, be sure to include enough volume as the error checking assumes only a single transfer. **Including a volume overage**. Be sure to include sufficient excess volume (dead volume) in the source well to ensure good accuracy and precision. For volume recommendations, see the *Labware Reference Guide* (workbench Literature Library page). The Starting volume in the source wells is the actual volume in the source well, which should equal the amount to be transferred plus the overage (dead volume). For 96 well plates, the Starting volume in the source wells setting is straightforward. For labware where multiple pipette tips can draw from a common source, the situation is more complex. The Starting volume is still the volume to be transferred plus the overage (dead volume) but the overage is not the overage for the entire common source. Instead, it is the overage for the common source divided by the number of virtual wells in that common source based on a 96 well plate map. • For example, a 12-Column Low-Profile Reservoir has 8 virtual wells per column so the overage used to calculate the Starting volume would be the recommended dead volume per column divided by 8. Similarly, an 8-Row Low-Profile Reservoir has 12 virtual wells per row so the overage used to calculate the Starting volume would be the dead volume per row divided by 12. • For a reformatting method where 50 µL is to be transferred per well with a 12-Column Low-Profile Reservoir as the source plate, the starting volume should be: 3 mL / 8 = 375 µL + 50 µL = 425 µL Default: 0 (µL) Range: 0–1050 (µL)

Input	Description
Pre-existing volume (µL) in destination wells	The initial volume in each well of the destination plate before the Reformatting utility is run. The software uses this volume for error checking, to calculate mixing volumes, and to calculate the liquid height in the wells to facilitate proper dynamic tip behavior. For 96 well plates, the Pre-existing volume in the destination wells is straightforward but for labware where multiple pipette tips can draw from a common source the situation is more complex. The Pre-existing volume is the total volume in the common source divided by the number of virtual wells in that common source, based on a 96-well plate map. For example, a 12-Column Low-Profile Reservoir has 8 virtual wells per column, so the Pre-existing volume would be the volume in the column divided by 8. Similarly, an 8-Row Low-Profile Reservoir has 12 virtual wells per row, so the Pre-existing volume would be the volume in the row divided by 12. Default: 0 (µL) Range: 0–995 (µL)
Source well mix cycles	The number of aspirate-and-dispense cycles used to mix the contents of the wells in the source plate before the sample is aspirated. Mix cycles are not recommended if any of the source wells will be used multiple times, because the mixing calculation assumes the volume in the source well is the Starting volume (µL) in source wells value. Therefore, following the initial volume drawn, the mixing volume could be greater than the volume in the well, and the mixing could introduce bubbles. The software assumes that all the wells in the source plate have the same volume. If any well has less volume than the volume set in the Starting volume (µL) in source wells field, mixing could introduce bubbles. For additional details, see "Automation movements and stepwise guidelines" on page 649. Default: 0 Range: 0–100
Destination well mix cycles	The number of aspirate-and-dispense cycles used to mix the contents of the wells in the destination plate after the sample is transferred to the destination well. The mixing volume may be too low for destination wells that receive multiple transfers because the mixing volume calculation assumes that the volume in the destination well is a single transfer plus the Pre-existing volume (µL) in the destination wells. If your protocol includes multiple source well transfers into a single destination well, Agilent recommends using a shaker to perform additional mixing. For additional details, see "Automation movements and stepwise guidelines" on page 649. Default: 0 Range: 0–100

Input	Description
Pre-wet tips	The option to wet the pipette tips with the liquid from the source well before drawing liquid from the source well. *Note:* Prewetting pipette tips is a common pipetting technique that can increase accuracy and precision in certain situations. For additional details, see "Automation movements and stepwise guidelines" on page 649. Default: Yes Options: Yes, No
Blowout volume (µL)	The volume of air to be drawn into the pipette tip before aspirating the sample. After dispensing the transferred sample, the volume of air remaining in the tip is dispensed (blown out) while the tip is still in the well. The blowout is followed by a tip touch on the east and west sides of the well. If the Blowout volume is set to 0, no blowout or tip touches will occur after the transfer is complete. For additional details, see "Automation movements and stepwise guidelines" on page 649. Default: 5 (µL) Range: 0–50 (µL)
Liquid class	The pipetting parameters (for example, aspiration and dispense speeds). The liquid class selection strongly influences the pipetting precision and accuracy. This choice only controls the aspiration and dispense of the samples. The mixing speed of the dilutions is fixed at 300 µL/sec. Options: • **<Automatic>** (default). Automatically assigns one of the following liquid classes, based on the volume being transferred. 0–20 µL (AM_250uLTipsLowVol) > 20 µL (AM_250uLTipsHighVol) These are good general-purpose liquid classes for most reagents that are used with the AssayMAP system. • **Slow Flow (5 µL/sec).** A slower flow rate is better for viscous solutions. • **Fast Flow (100 µL/sec).** A faster flow rate may improve performance for high organic solutions. If these flow rates do not provide the desired performance, you may enter a custom liquid class. To create a custom liquid class, see "To modify or create a custom liquid class:" on page 636. ***To enter a custom liquid class:*** In the **Liquid class** box, type the liquid class name exactly as it appears in the VWorks Liquid Library Editor. *Note:* The estimated run time (Status box area) may not be accurate for methods that use a custom liquid class.

Pipetting accuracy and liquid classes

IMPORTANT

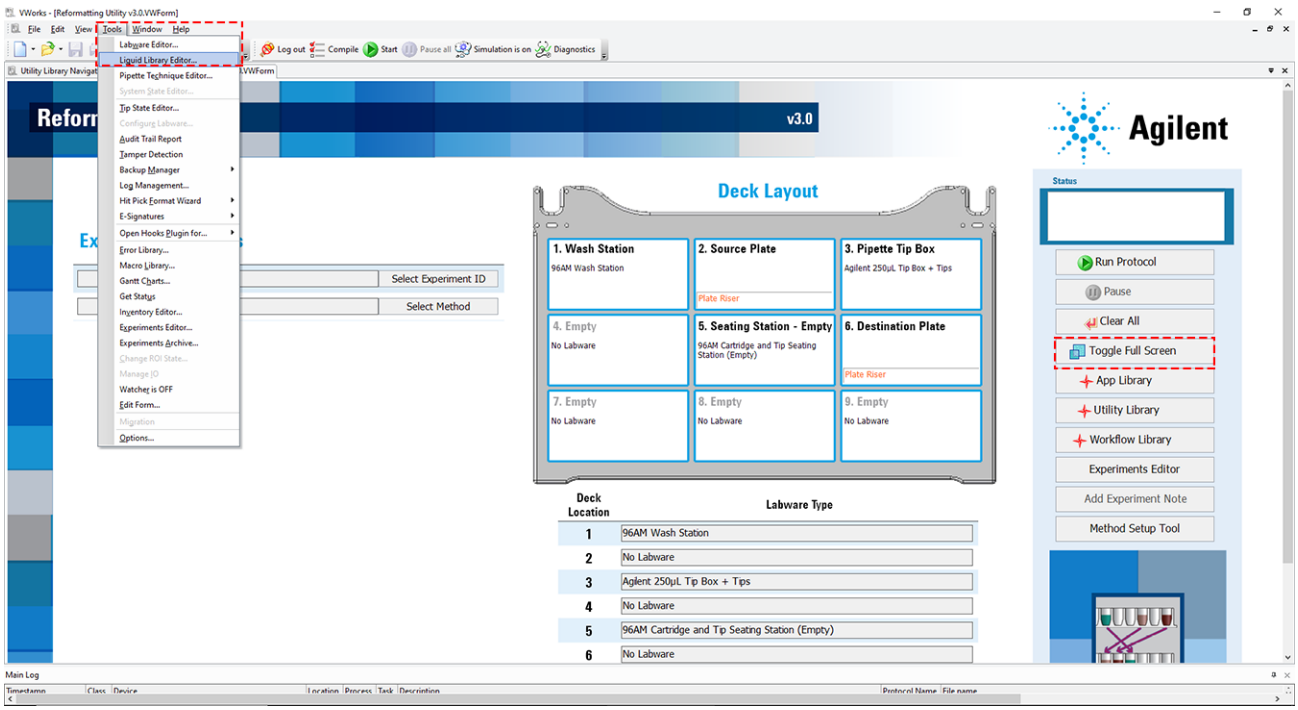
Agilent does not guarantee specific accuracy and precision results for the Reformatting v2.0 utility because accuracy and precision are too dependent on the composition and liquid properties of the solutions used.

Pipetting accuracy and precision can be greatly affected by liquid properties, such as viscosity and surface tension. If unacceptable accuracy and precision results are observed, you may modify these liquid classes to achieve acceptable results. Use the following procedure to modify or create a liquid class.

To modify or create a custom liquid class:

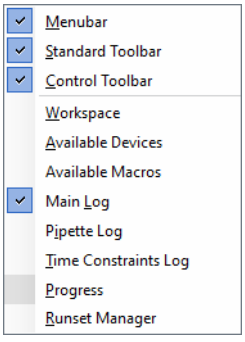
1 In the Reformatting form, click **Toggle Full Screen** to change the display of the form so that the VWorks menubar is visible.

Figure VWorks Tools menu and form displayed with full screen off



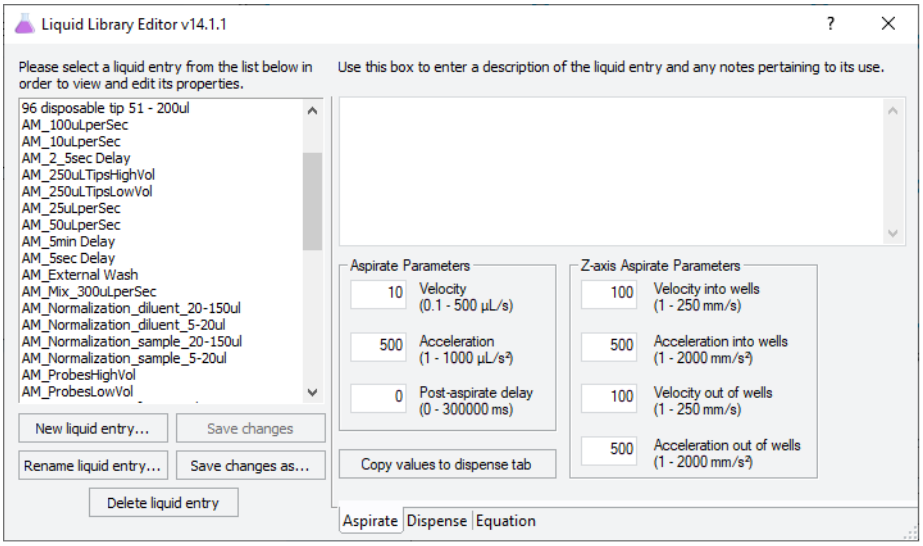
2 Click **Tools > Liquid Library Editor**. The Liquid Library Editor opens.

If the menubar is not visible in the window, right-click the window and select **Menubar** in the shortcut menu that appears.



3 For instructions on how to use the Liquid Library Editor, see the section on specifying pipette speed and accuracy in the *VWorks Automation Control Setup Guide*.

Figure VWorks Liquid Library Editor window



Step 3) Create list of samples to reformat

You can use any of the following options to create and edit the list of samples:

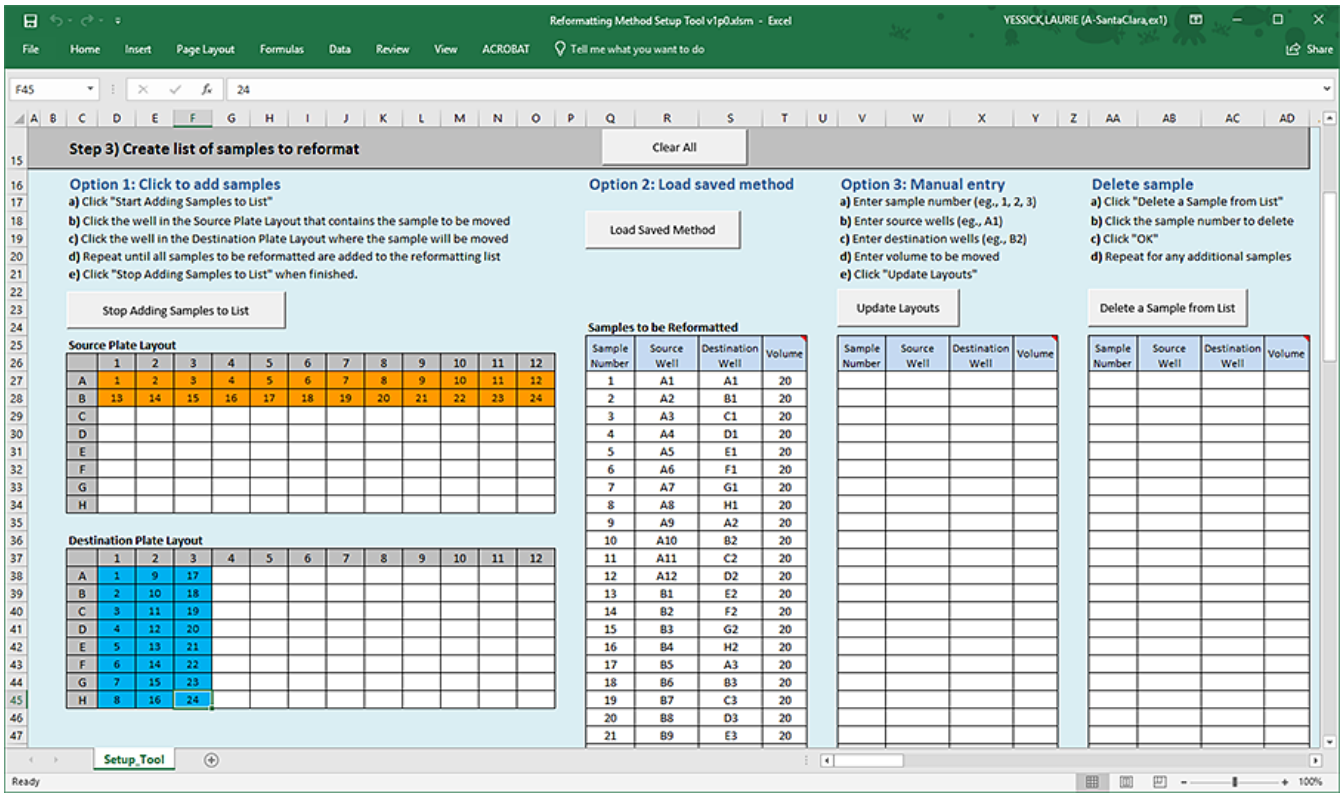
- Option 1. Click to add samples
- Option 2. Load saved method

  A saved method must be exported before it is available to load in Option 2. For details on how to export a method, see "Exporting and importing AssayMAP methods" on page 17.
- Option 3. Manual entry

You can also use a combination of options. For example, you can enter some sample transfers using option 1 or 2 and then you can add additional sample transfers manually. If you do this, remember to click **Update Layouts** after you are done.

The added samples appear in the Samples to be Reformatted table and in the Source Plate Layout and Destination Plate Layout, as the following figure shows. You can use the manual entry method to modify the volumes in the Samples to be Reformatted table.

Note: The Method Setup Tool assigns consecutive sample numbers based on the sequence in which you add the samples.



If necessary, you can delete samples from the list one at a time using the Delete sample feature. The Method Setup Tool consecutively renumbers the remaining samples in the list based on the sequence in which they were added.

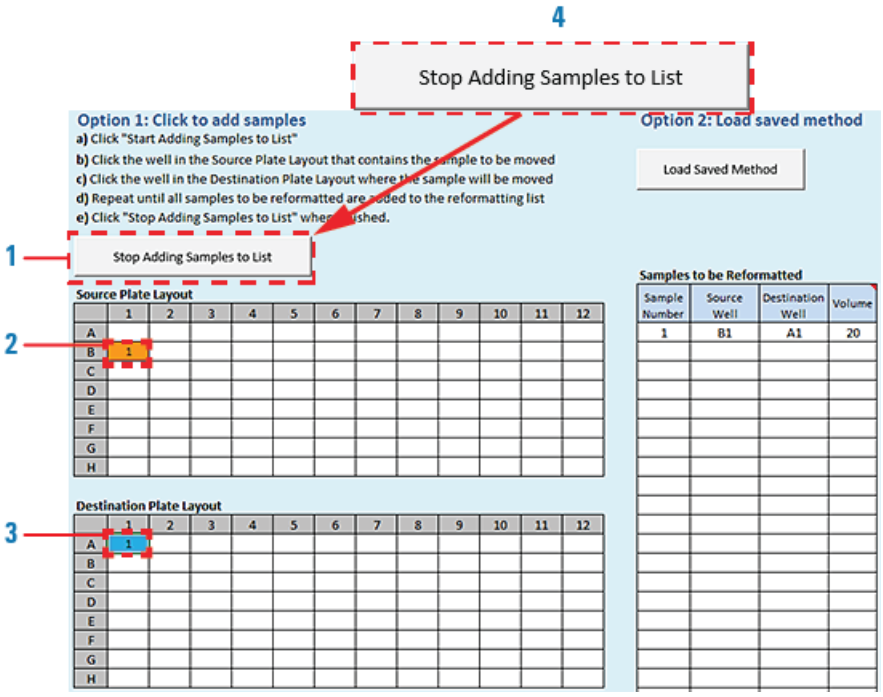
To use the Click to add samples option:

1. Click **Start Adding Samples to List**.

   Note: The button label changes to Stop Adding Samples to List.

2. In the **Source Plate Layout**, click the well that contains the sample to be moved, and then click the well in the **Destination Plate Layout** where this sample will go.

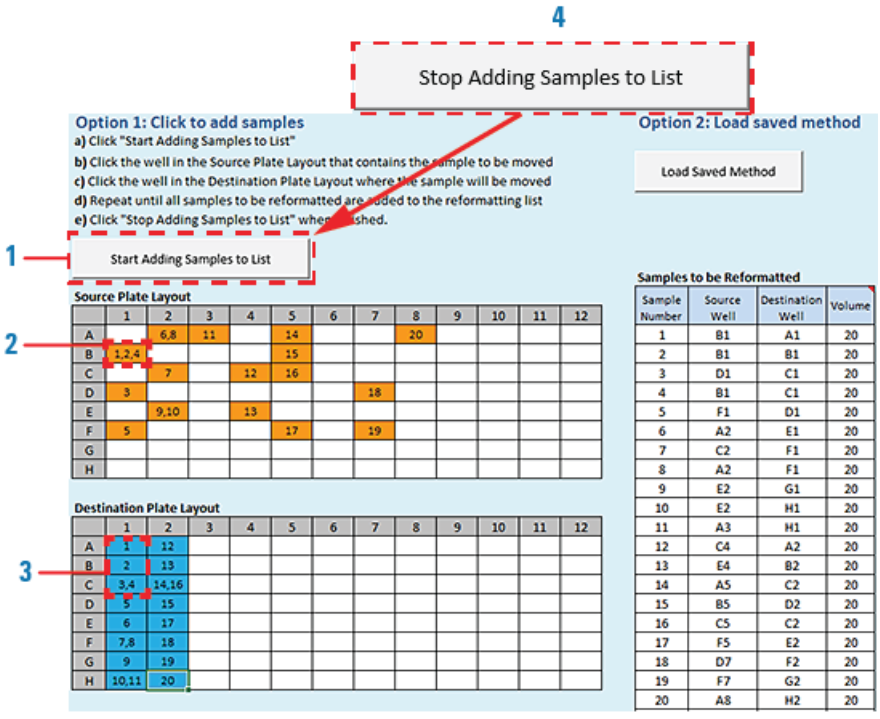
   As the figure shows, the cells change color in both plate layouts to designate the added samples. The corresponding sample and well designations appear in the Samples to be Reformatted table.



3 Repeat step 2 to add additional samples to the list.

- You can draw multiple times from the same source well. See B1 in the following source plate example.
- You can dispense multiple times in the same destination well. See C1, H1, and C2 in the following destination plate example.

4 When you are finished adding samples using Option 1, click **Stop Adding Samples to List**. See figure, item 4.



To use the Load Saved Method option:

1 Make sure you have exported a saved method:

   a Use the **File > Export Misc File** command in the VWorks window.

   b Ensure that the method file to be exported is a Reformatting v3.0 method.

   – VWorks Plus default storage location:
   … /VWorks Projects/VWorks/AM Methods/AM Reformatting v3.0

   – VWorks Standard default storage location:
   C:\OLSS Projects\VWorks Projects\VWorks\AM Methods\AM Reformatting v3.0

   For details, see "Exporting and importing AssayMAP methods" on page 17.

2 If applicable, click **Clear All** to refresh the tables in the Method Setup Tool.

3 Click **Load Saved Method**.

   a In the **Open** dialog box, select the exported method file (*.csv), and then click **Open**.

   b The samples appear in the Source Plate Layout, Destination Plate Layout, and Samples to be Reformatted table.

To use the Manual entry option:

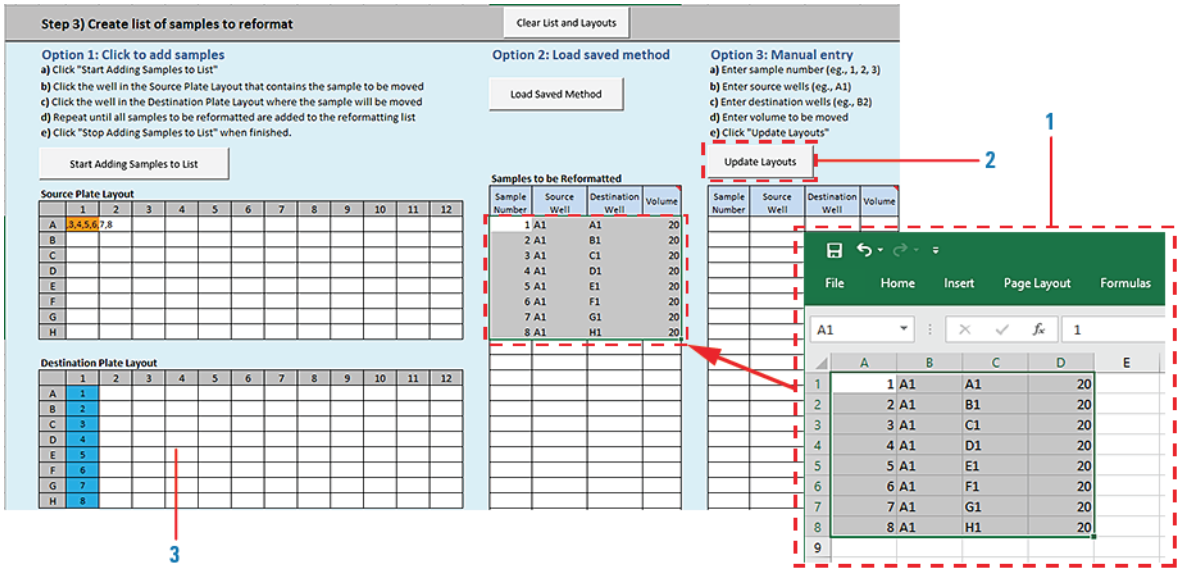
1 Type or copy-and-paste the required information in the **Samples to be Reformatted** table.

   Note: You can edit volumes that you entered using options 1 or 2. If the volume in the Samples to be Reformatted table has been modified so that it does not equal the volume set in Step 2, the corresponding cell turns yellow in the table. The yellow highlight is only a caution for you to verify that these values are intended.

   If you are copying rows from a table, ensure the content of the cells matches the column order of the **Samples to be Reformatted** table. See the following figure, item 1.

2 Click **Update Layouts** to populate the sample information in the Source Plate Layout and Destination Plate Layout (figure, item 2).

3 Notice that the cells change color in both plate layouts to designate the added samples (figure, item 3).



To delete a sample from the list:

1 Click **Delete a Sample from List**. See figure, item 1.

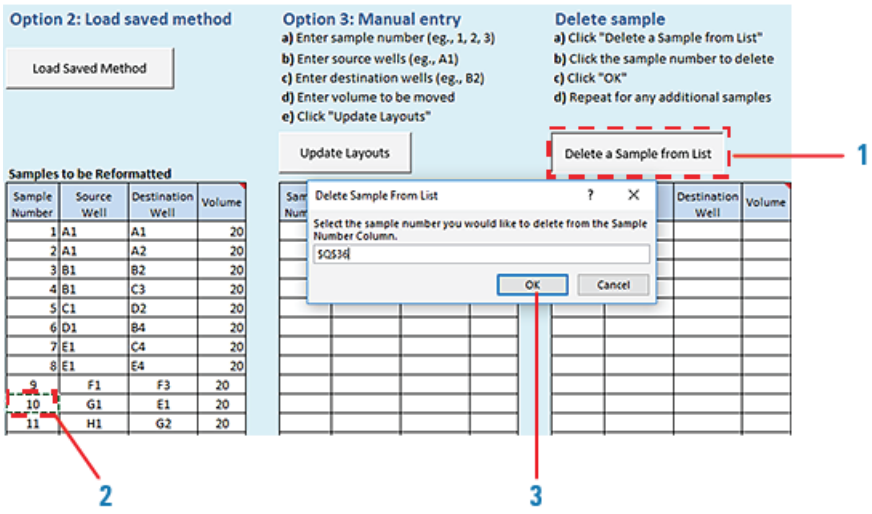
The Delete Sample From List dialog box opens.

2 In the **Samples to be Reformatted** table, click the cell in the **Sample Number** column (figure, item 2).

Note: Samples can be deleted only one at a time.

3 Click **OK** in the **Delete Sample From List** dialog box (figure, item 3).

The software updates the Samples to be Reformatted table and the plate layouts. If you delete a sample that is not at the end of the list, the software renumbers the samples remaining in the list in consecutive order.

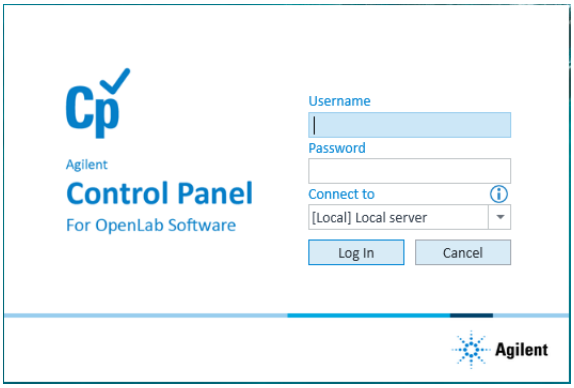


Step 4) Save method

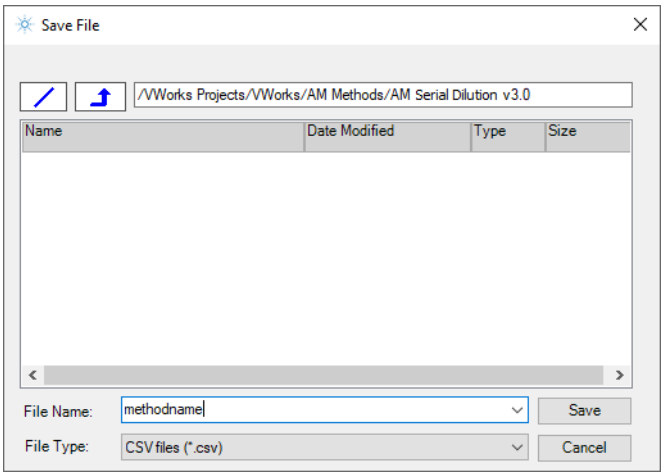
To save the reformatting method:

1 Click Create Method File.

2 In the login window, type your VWorks user name and password, and click **Log In**.



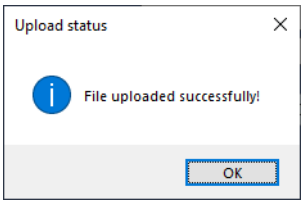
3 In the **Save File** dialog box, specify the file name and the storage location, and then click **Save**.



- VWorks Plus default storage location:
  … /VWorks Projects/VWorks/AM Methods/AM Reformatting v3.0
- VWorks Standard default storage location:
  C:\OLSS Projects\VWorks Projects\VWorks\AM Methods\AM Reformatting v3.0

Note: The method file is stored as a record of interest (ROI) in an archive (.csv.roiZip). VWorks cannot load files (.roiZip extension) that have been modified or renamed outside of the Protein Sample Prep Workbench or VWorks software.

4 Click **OK** when the uploaded successfully message appears. The Method Setup Tool closes.



To display the method's labware selections and preparation instructions do one of the following:

- Open the Reformatting utility and select the method. For details, see "Running the protocol" on page 643.
- Export the method and view it in Microsoft Excel. For details, see "Exporting and importing AssayMAP methods" on page 17.

Running the protocol

The Reformatting protocol does the following:

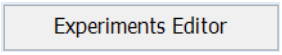
- Washes and dries the syringes.
- Transfers sample from a 96-well source plate to a 96-well destination plate

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking

  in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

- Prepare the reformatting setup method. See "Setting up a Reformatting method" on page 628.
- Display the method's labware selections and preparation instructions by doing one of the following:
  - Open the Reformatting utility and select the method. For details, see "Setting up the protocol" on page 644.
  - Export the method and view it in Microsoft Excel. For details, see "Exporting and importing AssayMAP methods" on page 17.

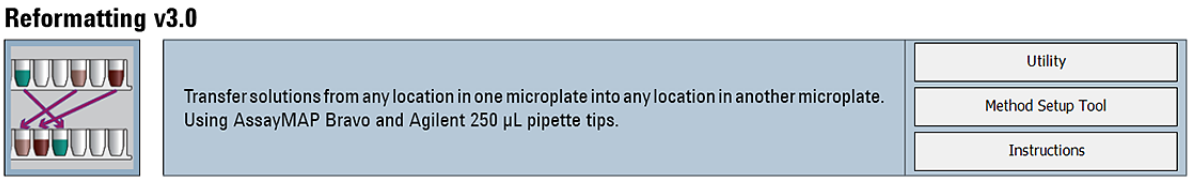
- Prepare the source and destination plates. Ensure that the type of labware and volumes per well match what is specified in the reformatting method.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- If necessary, run the Startup protocol to initialize the AssayMAP Bravo Platform and prime the wash lines. See the System Startup/Shutdown v3.0 User Guide utility.

IMPORTANT To minimize evaporation, fill the labware immediately before run time or keep them covered until you run the protocol.

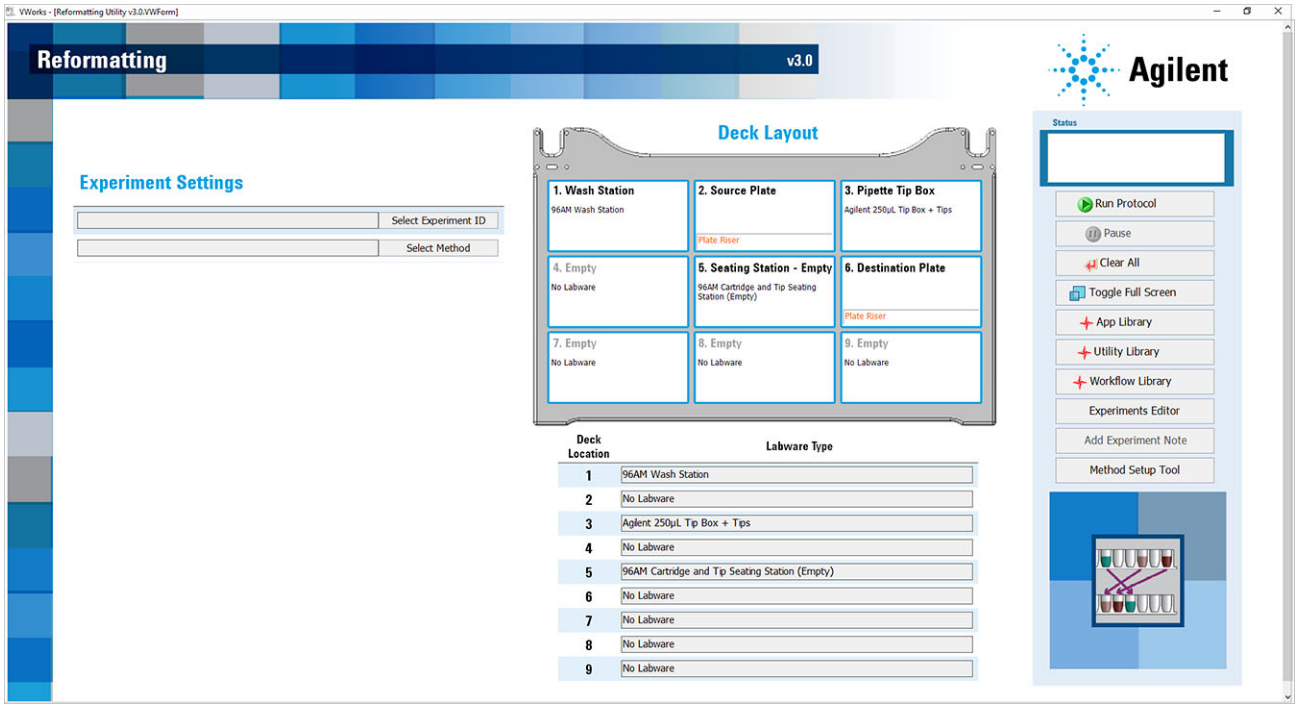
Setting up the protocol

To set up the protocol:

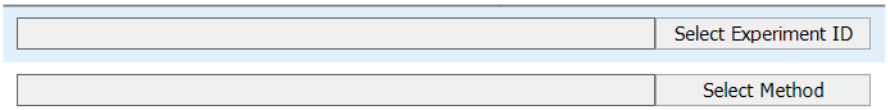
1 Locate the **Reformatting** banner in the **Utility Library**, and then click **Utility**.



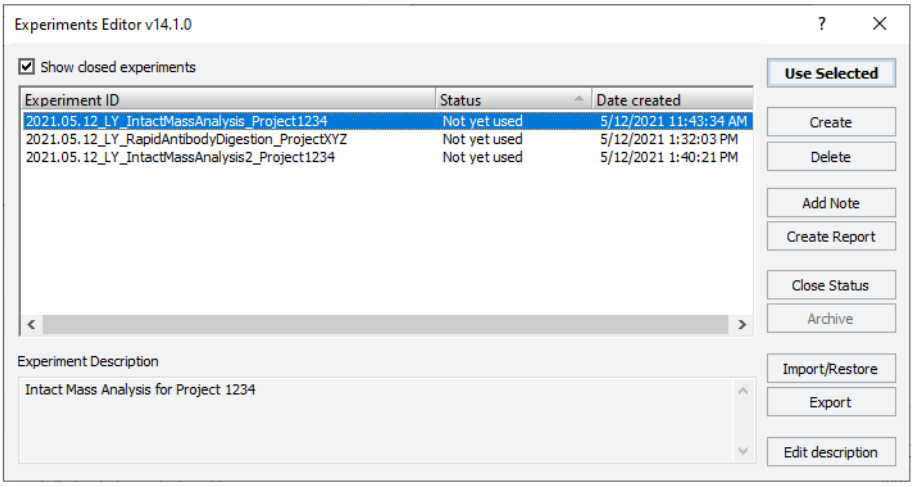
The Reformatting utility opens.



2 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



3 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

4 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

The form displays the method labware selections and preparation instructions.

- To run the selected method, go to "Starting the protocol run" on page 645.
- To create or modify a method, see "Setting up a Reformatting method" on page 628.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. You must use pipette tips for the mock run.

Starting the protocol run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

To start the protocol run:

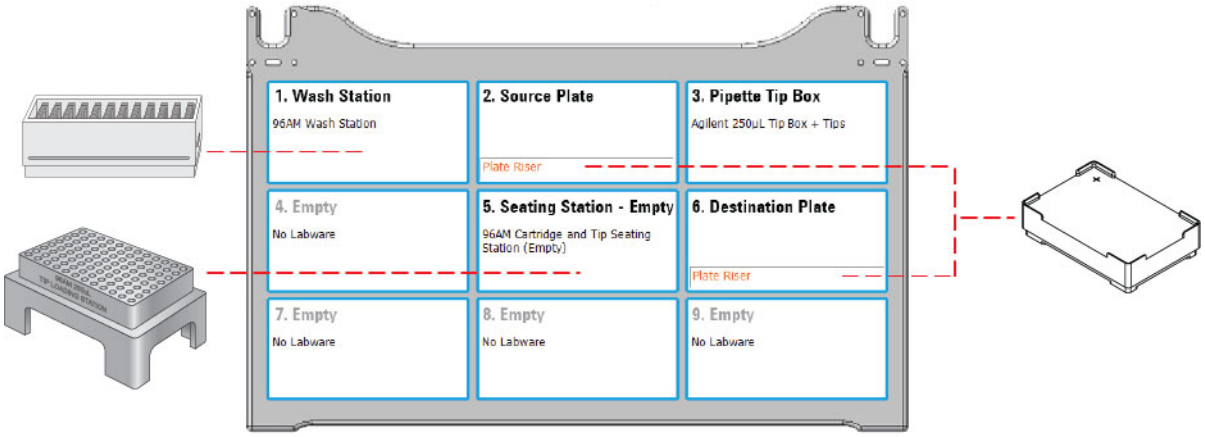
1 Ensure that the accessories and labware are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

Make sure the labware are properly seated on the Bravo deck.

- The Bravo Plate Risers are securely in place at deck locations 2 and 6. The source plate is seated on the Bravo Plate Riser at deck location 2. The destination plate is seated on the Bravo Plate Riser at deck location 6.
- The tip box full of fresh pipette tips is at deck location 3 and the empty 96AM Cartridge & Tip Seating Station is at deck location 5.

  Note: A full tip box is required for each run even if the run uses less than 96 pipette tips.

  Note: The protocol will transfer the pipette tips to the seating station.

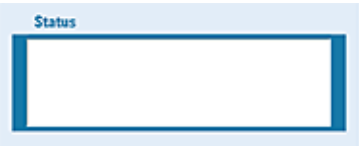


CAUTION **To prevent a potential collision, ensure that no thermal plate insert is on the Peltier Thermal Station installed at deck location 4.**

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



Note: The run time varies depending on the method. The software updates the run time throughout the course of the run, and the completion time estimate becomes more and more accurate as the run progresses.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

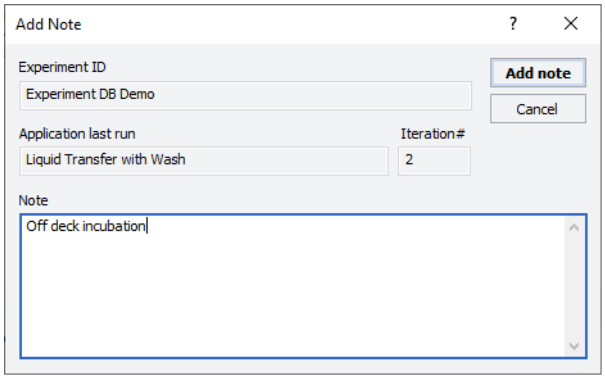
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

To clean up after a run:

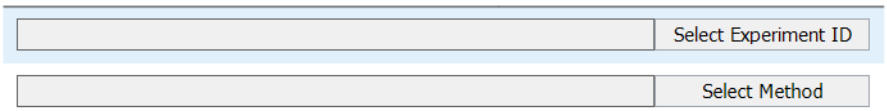
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

a Open the **Syringe Wash** utility  .

b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines

This topic provides guidelines for adjusting the protocol, and notes about the application.

Note: For maximum well capacities and recommended excess volumes in the source plate and destination plate, see the *Labware Reference Guide* in the Literature Library page of the workbench.

Automation movements and stepwise guidelines

Protocol step	Head moves to deck location...	Action
1. Syringe Wash	1	Performs 1 external syringe wash at the wash station.
2. Syringe Drying	1	Performs 4 syringe aspirate-and-dispense cycles above the wash station to cycle air in and out of the syringes. The syringes move over the chimneys after each cycle to remove any droplets that were pushed out of the syringes during the cycle.
3. Initial Tip Transfer	3	Presses on all the 250-µL pipette tips from the tip box. *Note:* The tip box must be full even when using fewer than 96 pipette tips because of the tip-seating force that is used for this step.
	5	Ejects the pipette tips into the seating station.
4. Single Tip Pickup	5	Positions the head at an offset from the seating station to use probe A12 to pick up the next available individual pipette tip, starting at H1.

Protocol step	Head moves to deck location…	Action
5. Prep for Transfer	2	Positions the head at an offset to the source plate so that the A12 pipette tip moves to the designated well. • If a blowout volume is specified, aspirates an air gap. This volume is used to ensure that no liquid remains in the pipette tip after the contents of the tip are dispensed. A small blowout volume can help improve precision and accuracy. The magnitude of this effect is usually small for aqueous solutions but can be significant with viscous solutions. Default: 5 µL Range: 0-50 µL *Note:* The size of the air gap reduces the possible pipetting volume per pipetting cycle. Larger air gaps have diminishing returns in terms of improved accuracy and precision. The relationship between the size of the air gap and the precision and accuracy is highly dependent on the solution being moved. Agilent recommends starting with the default value to determine if any change is required. • If specified, prewets the A12 pipette tip in the designated source well. Prewetting pipette tips is best practice for good pipetting accuracy and precision. Default: Yes Options: Yes, No • If specified, mixes the sample in the source well the specified number of cycles. The volume used for the mixing process is automatically calculated using the following formula. Mixing formula: – If (x - 50 µL) > Tip Capacity, then Mixing Volume = Tip Capacity – If 50 µL ≤ x ≤ Syringe/Tip Capacity, then Mixing Volume = 0.75x – If x < 50 µL, then Mixing Volume = 0.5x where, x is the final volume in the destination wells, and maximum 250 µL Tip Capacity = 140 µL

Protocol step	Head moves to deck location...	Action
		Note: – The mix cycle default is set at 0 to ensure that mixing is not done unintentionally. If samples are centrifuged before reformatting, mixing would not be wanted. – Generally, aqueous solutions are well mixed by 5 cycles if the volume is <200 µL. More viscus and larger volume solutions require more mix cycles, which must be determined empirically. – The more mix cycles you use, the longer the run time. **IMPORTANT** Mix cycles are not recommended if any of the source wells will be used multiple times, as the mixing calculation assumes the volume in the source well is the Starting volume (µL) in source wells value. Therefore, after aspirating the initial volume, the mixing volume could be greater than the volume remaining in the well, and the mixing could introduce bubbles. **IMPORTANT** The software assumes that all the wells in the source plate have the same volume. If any well has less volume than the volume set in the Starting volume (µL) in source wells field, mixing could introduce bubbles.
6. Transfer	2	Aspirates the specified volume from the source plate well into the A12 pipette tip.

Protocol step	Head moves to deck location…	Action
	6	Positions the head at an offset to the destination plate so that the A12 pipette tip moves to the designated well. • Dispenses the sample into the destination well. • If specified, mixes the sample in the well the specified number of cycles. The volume used for the mixing process is automatically calculated using the following formula. Mixing formula: – If (x - 50 µL) > Tip Capacity, then Mixing Volume = Tip Capacity – If 50 µL ≤ *x* ≤ Syringe/Tip Capacity, then Mixing Volume = 0.75*x* – If *x* < 50 µL, then Mixing Volume = 0.5*x* where, *x* is the final volume in the destination wells, and maximum 250 µL Tip Capacity = 140 µL *Note:* – The mix cycle default is set at 0 to ensure that mixing is not done unintentionally, and is typically only done if there is a pre-existing volume in the destination well. – Generally, aqueous solutions are well mixed by 5 cycles if the volume is <200 µL. More viscus and larger volume solutions require more mix cycles, which must be determined empirically. – The more mix cycles you use, the longer the run time. The mixing volume may be far too low for destination wells that receive multiple transfers because the mixing volume calculation assumes that the volume in the destination well is the single transfer volume plus the pre-existing volume in the destination well. • If a blowout volume is specified, the head raises to near the top of the well, and the specified volume of air is dispensed followed by a tip touch on the east and west sides of the well.
7. Single Tip Eject	3	Ejects the used pipette tip into the tip box
8. Additional Transfers	multiple	Repeats processes 3 through 6 for every designated well in the source plate.
9. Park Head	1	Moves to a safe height above the wash station.

18 Serial Dilution v3.0 User Guide

This chapter contains the following topics:

Utility description

Serial Dilution v3.0. This utility enables you to automatically generate serial dilutions in a 96-well microplate. You create a method using the Serial Dilution Method Setup Tool. The utility uses your method to transfer the appropriate volume of diluent and source sample into the designated wells of the serial dilution plate to create the desired dilution series.

The serial dilution method specifies:

- Whether the dilution series progresses by columns or rows and the direction of the series (such as, top to bottom, bottom to top, left to right, or right to left)
- The number of steps in the dilution series and the number of dilution replicates (2 to 24 dilutions and 1 to 5 replicates)
- The final volume of the serial dilution (5–500 µL)
- If the utility will change pipette tips after every dilution step, or use the same pipette tips throughout the dilution series
- Liquid-handling parameters, including the number of mix cycles after every dilution, whether to prewet the pipette tips, the blowout volume, and the choice of liquid class
- If a constant volume (for example, internal standard) will be added to the dilution series after running the utility

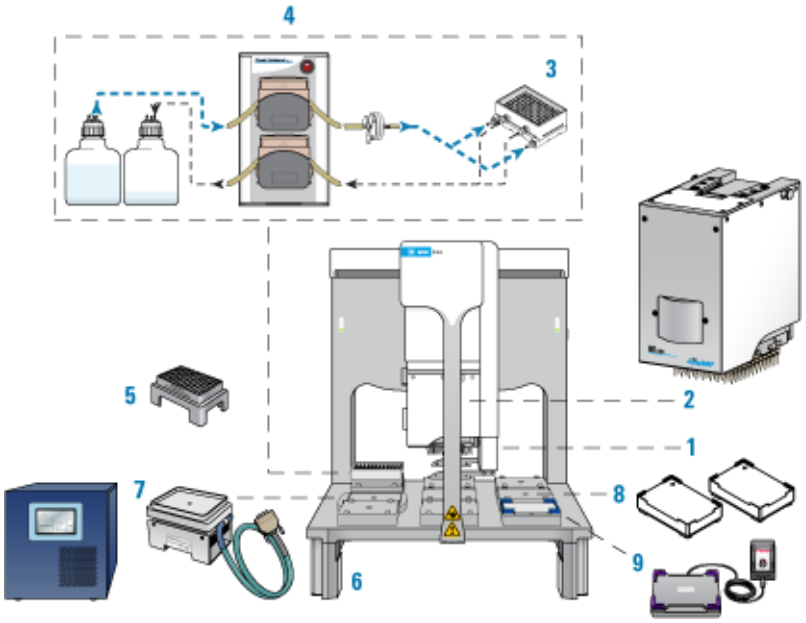
Before you start

This topic lists the required hardware, software, labware, and solutions for running the Serial Dilution protocol. If you have questions about these items, contact Agilent Customer Service.

Hardware

The AssayMAP Bravo Platform is required for running the AssayMAP protocols. The following figure and table describe the platform components.

Figure AssayMAP Bravo Platform components



Item	Required hardware
1	Gripper upgrade
2	Bravo 96AM Head
3	96AM Wash Station
4	Pump Module 2.0 and two carboys
5	96AM Cartridge & Tip Seating Station
6	Bravo Risers, 14.6 cm
7	Peltier Thermal Station with STC controller
8	Plate Risers, 2.84 cm (two)
9	Orbital Shaking Station with Control Unit

Optional equipment. The following equipment is recommended when preparing the samples and reagents:

- Microplate centrifuge, such as the Agilent Microplate Centrifuge or equivalent
- Microplate sealer, such as the Agilent PlateLoc Thermal Microplate Sealer or equivalent

Software

The following table lists the minimum software requirements.

Software	Version
Agilent VWorks Plus (compliance-enabled edition) or VWorks Standard	14.1.1
Agilent Protein Sample Prep Workbench	4.0
Microsoft Excel Required for the reagent volume calculators and method setup tools.	Microsoft Office 365 32-bit edition

For an overview of the software components, see "Overview of software architecture" on page 15.

Labware

The Serial Dilution protocol requires the following labware:

- Full tip box of 96 250-µL pipette tips, Agilent 19477-002
- Labware for the diluent reservoir and serial dilution plate

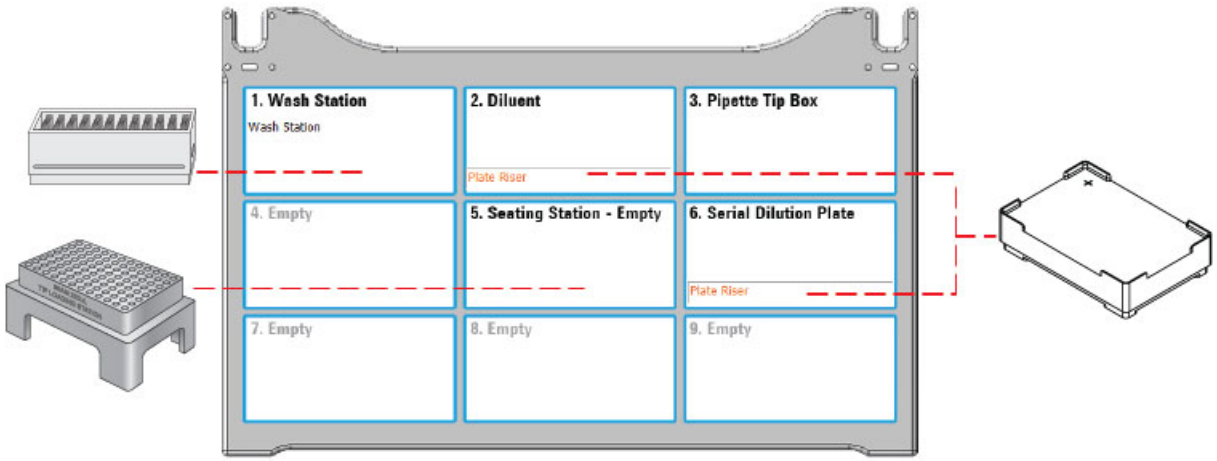
The following table provides a complete list of the labware options and the corresponding deck locations.

The following figure shows the nine Bravo deck locations for labware.

CAUTION **Using different pipette tips, such as filtered tips, will result in equipment damage. Ensure that you use the standard 250-µL pipette tip (Agilent 19477-002).**

CAUTION **Use only the labware specified for each deck location. Using different labware or placing labware at unapproved deck locations can cause a collision resulting in equipment damage.**

Figure Accessory and labware locations on the Bravo deck (top view)



Labware	Manufacturer part number*	Deck location options
8 Row, Low Profile Reservoir, Natural PP	Agilent 201282-100	2
12 Column, Low Profile Reservoir, Natural PP	Agilent 201280-100	2
96 V11 Manual Fill Reservoir	Agilent G5498B#049	2
Reservoir, Seahorse 201254-100, PP, no walls, pyramid bottom	Agilent 201254-100	2
96 AbGene 1127, 1mL Deep Well, Square Well, Round Bottom	ABgene AB-1127	2, 6
96 Costar 3363, PP Conical Bottom	Corning Costar 3363	2, 6
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	Eppendorf 30129300	2, 6
96 Eppendorf 96-500_V-bottom, Clear PolyPro	Eppendorf 96/500	2, 6
96 Bio-Rad PCR, Hard-Shell, Low-Profile, Full Skirt	Bio-Rad HSP-9611	2, 6
96 Greiner 650201_U-Bottom, Clear PolyPro	Greiner 650201	2, 6
96 Greiner 650207_U-Bottom, White PolyPro	Greiner 650207	2, 6
96 Greiner 651201_V-Bottom, Clear PolyPro	Greiner 651201	2, 6
96 Greiner 652270, PCR, Full Skirt, PolyPro	Greiner 652270	2, 6
96 Greiner 675801, Half Area, Flat-Bottom, UV Star	Greiner 675801	6
96 Thermo Matrix 3732, V-bottom, 0.75ml Storage Tubes	Thermo Fisher Scientific 3732	2, 6
96 Thermo Matrix 3735, V-bottom, 500µl Storage Tubes	Thermo Fisher Scientific 3735	2, 6
96 Thermo Matrix 3744, V-bottom, 500µl ScrewTop Storage Tubes	Thermo Fisher Scientific 3744	2, 6
96 Waters 186005837, Clear PolyPro	Waters 186005837	2, 6

*For dimensionally equivalent alternatives and other details about the labware, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

The unusable volume (also known as *dead* or overage volume) is the volume that cannot be reliably aspirated from the microplate well due to pipetting limitations that arise from differences in well-bottom geometry. The following table provides some general guidelines on how different microplate geometries can affect this value.

Table Guidelines on how microplate geometry can affect volume requirements

Well-bottom geometry	Examples	Unusable volume expectations
Flat bottom	Greiner 675801	Generally, the worst plates for minimizing dead volume. Liquids are attracted to the intersection of the plate bottom and side walls.
Round (U) bottom	Greiner 650201, Greiner 650207	Generally, good options for minimizing dead volume. A round bottom forces liquid to collect in the center of the well.
V-bottom and PCR	• V-Bottom: Greiner 651201, Corning Costar 3363 • PCR Plate: Eppendorf 30129300, Greiner 652270	Generally, the best plates for minimizing dead volume. Steep side walls force liquid to collect in the center of the well, making it easy to access.

For volume overage recommendations by labware type, see the *Labware Reference Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Solutions

The Serial Dilution utility requires the following solutions:

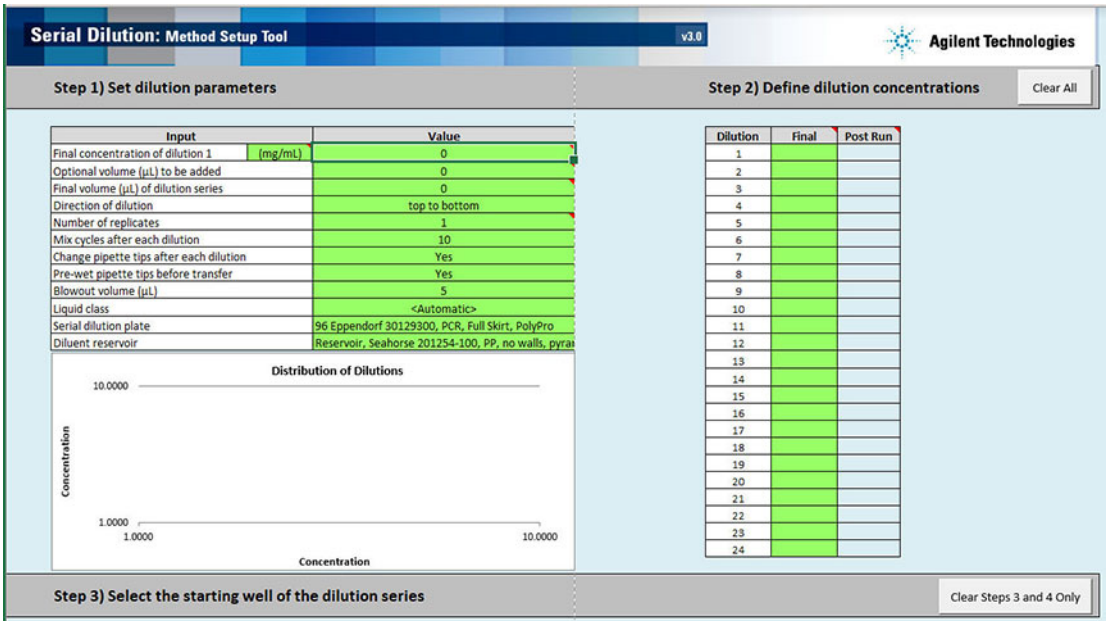
- Diluent to adjust the sample concentration
- Purified water for the wash station

Setting up a Serial Dilution method

The Method Setup Tool is a Microsoft Excel-based tool that steps you through the process of creating and saving a method file that contains all the information required to run the Serial Dilution protocol on the AssayMAP Bravo Platform. This tool uses formulas to calculate volume requirements for the samples and diluent based on your input.

Note: When you select a method in the Serial Dilution utility, the form displays the corresponding labware selections and diluent preparation instructions.

Figure Serial Dilution Method Setup Tool

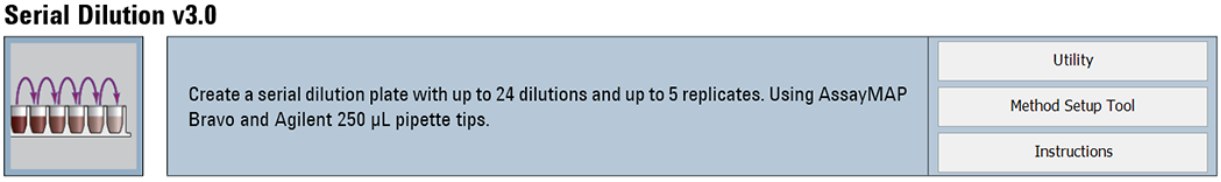


Opening the Method Setup Tool

You can open the tool from the Utility Library or from the Serial Dilution form.

To open the Method Setup Tool:

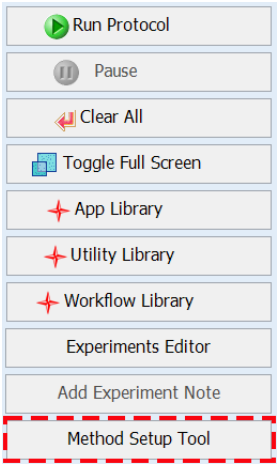
1 In the **Utility Library**, locate the **Serial Dilution** banner.



2 Click one of the following buttons:

- **Method Setup Tool**. Microsoft Excel starts and displays the Method Setup Tool.
- **Utility**. The Serial Dilution utility opens.

  In the navigation pane, click **Method Setup Tool**. Microsoft Excel starts and displays the Method Setup Tool.



IMPORTANT

In Microsoft Excel, ensure that you enable content.

Overview of steps in Method Setup Tool

The Method Setup Tool has 6 distinct steps. The following table provides an overview of the steps.

Table Overview of steps in Method Setup Tool

Step		Description	
1	Set dilution parameters	Enter values for the following inputs:	Default value (Range)
		Final concentration of dilution 1	0 (>0)
		Optional volume (µL) to be added	0 (0 to < Final volume of dilution series)
		Final volume (µL) of dilution series	0 (5–500)
		Direction of dilution	top to bottom (top to bottom, bottom to top, left to right, right to left)
		Number of replicates	1 (1–5)
		Mix cycles after each dilution	10 (0–100)
		Change pipette tips after each dilution	Yes (No/Yes)
		Pre-wet pipette tips before transfer	Yes (No/Yes)
		Blowout volume (µL)	5 (0–50)
		Liquid class	Automatic (preset or custom)
		Serial dilution plate	"Labware" on page 656
		Diluent reservoir	"Labware" on page 656
2	Define dilution concentrations	Enter the final target concentration for each serial dilution step.	
3	Select the starting well of the dilution series	Click **Select Starting Well**, and then click the cell in the **Serial Dilution Plate** map where the replicate 1 of dilution 1 will be located. The setup tool will display the plate layout based on the input provided in steps 1 and 2.	
4	Calculate the sample and diluent volumes	Click **Calculate Volumes**. The setup tool calculates the required volumes of sample and diluent based on the input provided in steps 1 to 3.	
5	Correct these errors	Resolve any errors that the setup tool highlights.	

Step		Description
6	Create the method file	Click **Create Serial Dilution Method**. The setup tool creates a comma-separated value (.csv) text file that you use to run the utility. After you save the method, the Method Setup Tool closes. When you select the method in the Serial Dilution utility, the form displays the corresponding labware selections and diluent preparation instructions. Follow the instructions that appear in the form to prepare the serial dilution and diluent plates.

IMPORTANT

If you change anything in step 1 or 2 after completing step 3 or 4, make sure that you click **Clear Steps 3 and 4 Only** and redo steps 3 and 4.

Step 1) Set dilution parameters

Note: Only the cells highlighted in green are editable.

To set the dilution parameters:

1. To clear any data from the Serial Dilution Method Setup Tool, click **Clear All** (upper right corner).
2. Specify the following values in the green cells:

   For additional details on these settings, see "Assay development guidelines" on page 679.

Input	Description
Final concentration of dilution 1	
Concentration units	In the Input column, verify that the unit of measure for the sample concentration is correct. If necessary, type the correct units (default is mg/mL). This input is used to populate other sections of the form. This value has no impact on the subsequent data or volume calculations.
Concentration value	In the Value column, type the final concentration of the most concentrated level in the serial dilution series (e.g., Dilution 1 in Step 2).

Input	Description
Optional volume (µL) to be added	The volume to be added manually or using the Reagent Transfer utility after the Serial Dilution run, for example, an internal standard. The set up tool uses this value to reduce the volume of the serial dilution to the final volume minus the optional volume to be added. The set up tool also uses this value to increase the concentration of each serial dilution step (see Post Run cells in Step 2) so that when the optional volume is added, the final serial dilution volumes and concentrations match the desired final serial dilution series volumes and concentrations. **IMPORTANT** The value you enter here must be less than the Final volume. The recommended minimum volume is 5 µL. For example, if the Final volume = 50 µL and Optional volume to add later = 10 µL, the Serial Dilution steps will have a volume of 40 µL after the run, at which point the optional 10 µL will be added for a total volume of 50 µL. Default: 0 (µL) Range: 0–50 (µL)
Final volume (µL) of dilution series	The final volume of each step in the dilution series. This includes the Optional volume (µL) to be added. *Note:* The practical range is approximately 20–500 µL, depending on the number and concentration of the dilution steps. The high end of the range is limited by well capacities and the high volume transfers required during the early steps of the dilution series compared to the final volume of the dilution steps. The low end of the range is limited by the requirement that the transfers be 5 µL or greater to ensure high precision and accuracy. Large-volume dilution series require more mix cycles (up to 100 for the largest volume serial dilutions) because of the limited volume that can be used for each mix cycle. The results for large volume dilution series may not be as accurate as smaller volume dilution series. Default: 0 (µL) Range: 5–500 (µL)
Direction of dilution	The direction in which the dilution series should progress. The dilution series follows a serpentine pattern. See the example in "Step 3) Select the starting well of the dilution series" on page 668. Default: top to bottom Options: top to bottom, bottom to top, left to right, right to left

Input	Description
Number of replicates	The number of replicates of each dilution step in the dilution series. *Note:* This cell turns yellow if you select 5 replicates. The yellow cautions you that the serial dilution may not fit on the serial dilution plate, depending on the number of steps in the dilution series. The maximum number of dilution steps with 5 replicates is as follows: • 16 for top-to-bottom or bottom-to-top dilution series • 12 for left-to-right or right-to-left dilution series The Number of replicates value determines the total number of wells that will be prepared for each step in the dilution series. For example, if the number of replicates is set to 2, then on run completion, there will be two wells containing each concentration in the series. Default: 1 Range: 1–5
Mix cycles after each dilution	The number of aspirate-and-dispense cycles used to mix the contents of the wells in the serial dilution plate. Default: 10 Range: 0–100
Change pipette tips after each dilution	The option to change pipette tips before transferring the sample for each dilution step. Default: Yes Options: Yes, No
Pre-wet pipette tips before transfer	The option to wet the pipette tips with the sample before aspirating the sample. *Note:* Prewetting the pipette tips is a common pipetting technique that can increase accuracy in certain situations. Default: Yes Options: Yes, No
Blowout volume (µL)	The volume of air to be drawn into the pipette tips before aspirating the sample. After dispensing the transferred sample, the volume of air remaining in the pipette tips is dispensed (blown out) while the tips are still in the wells. The blowout is followed by a tip touch on the east and west sides of the well. If the Blowout volume is set to 0, no blowout or tip touches will occur. Default: 5 (µL) Range: 0–50 (µL)

Input	Description
Liquid class	The pipetting parameters (for example, aspiration and dispense speed). The liquid class selection strongly influences the pipetting precision and accuracy. This choice only controls the aspiration and dispense of the samples and diluent. The mixing speed of the dilutions is fixed at 300 µL/sec. Options: • **<Automatic>** (default). Automatically assigns one of the following liquid classes, based on the volume being transferred. 0–20 µL (AM_250uLTipsLowVol) > 20 µL (AM_250uLTipsHighVol) These are good general-purpose liquid classes for most reagents that are used with the AssayMAP system. • **Slow Flow (5 µL/sec).** A slower flow rate is better for viscous solutions. • **Fast Flow (100 µL/sec).** A faster flow rate may improve performance for high organic solutions. If these flow rates do not provide the desired performance, you may enter a custom liquid class. To create a custom liquid class, you use the VWorks Liquid Library Editor. For details on how to open the VWorks Liquid Library Editor, see "To create a custom liquid class:" on page 666. ***To enter a custom liquid class:*** In the **Liquid Class** box, type the liquid class name exactly as it appears in the VWorks Liquid Library Editor. *Note:* The estimated run time (Status box area) may not be accurate for methods that use a custom liquid class.
Serial dilution plate	The labware that you are placing on the plate riser at deck location 6. For options, see "Labware" on page 656. For the capacity limits and the volume overage recommended for each labware, see the *Labware Reference Guide* in the Literature Library page of the workbench. *Note:* The intermediate volumes required to generate the serial dilution will be larger than the final volume of the serial dilution. If you have chosen a plate type whose capacity is lower than required, you will be alerted in steps 4 and 5.
Diluent reservoir	The labware that you are placing on the plate riser at deck location 2. For options, see "Labware" on page 656. For the capacity limits and the volume overage recommended for each labware, see the *Labware Reference Guide* in the Literature Library page of the workbench. Be sure to choose a diluent reservoir that can hold the volume required for the serial dilution. The volume of diluent and the location where it should be placed will be given in step 7 of the Method Setup Tool. The plate type chosen must have enough well capacity to hold the dilution indicated in step 7 plus the required well overage. For volume guidelines, see "Preparing the diluent and samples" on page 672. *Note:* If the selected labware cannot hold the volume of diluent required for the serial dilution, an error message will appear in steps 4 and 5.

Pipetting accuracy and liquid classes

IMPORTANT

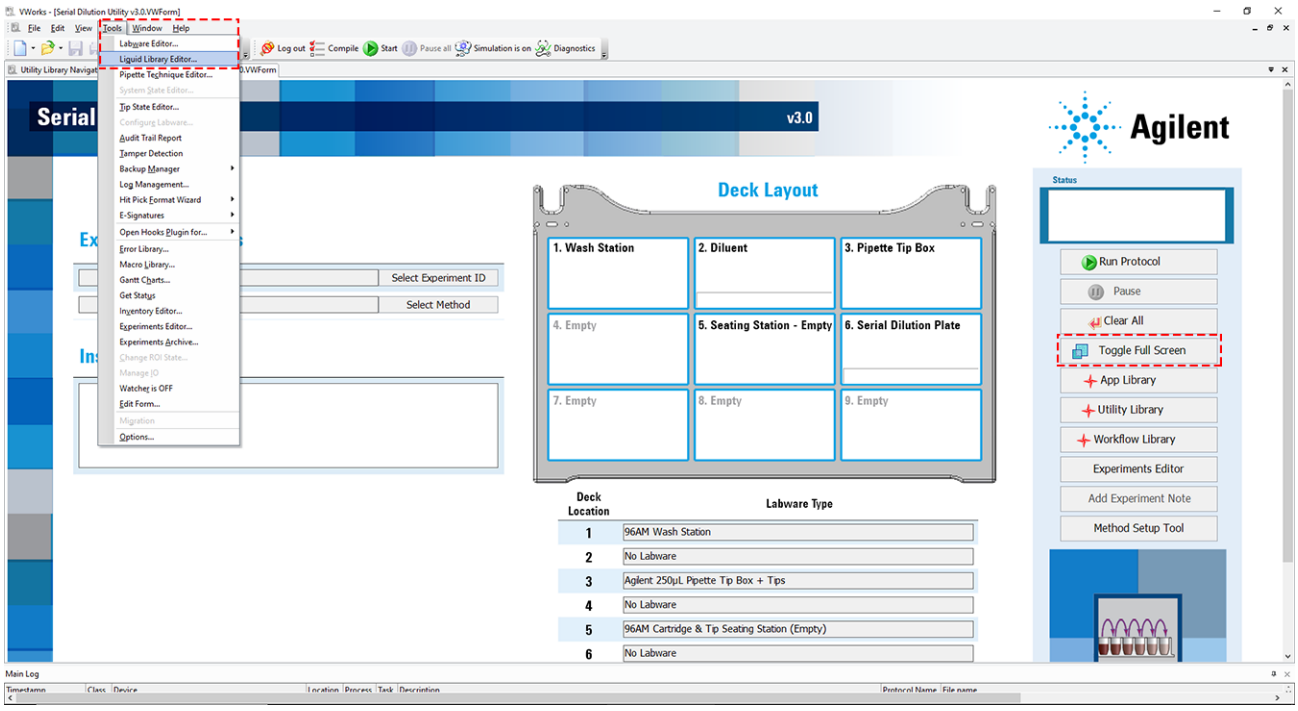
Agilent Technologies does not guarantee specific accuracy and precision results for the Serial Dilution v2.0 utility because accuracy and precision are too dependent on the composition and liquid properties of the solutions used.

Pipetting accuracy and precision can be greatly affected by liquid properties, such as viscosity and surface tension. If unacceptable accuracy and precision results are observed, you may modify these liquid classes to achieve acceptable results. Use the following procedure to modify or create a liquid class.

To create a custom liquid class:

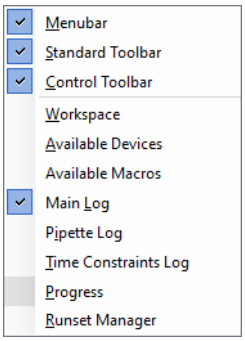
1 In the **Serial Dilution** form, click **Toggle Full Screen** to change the display of the form so that the VWorks menubar and toolbar are visible, as the following figure shows.

Figure VWorks Tools menu and form displayed with full screen off



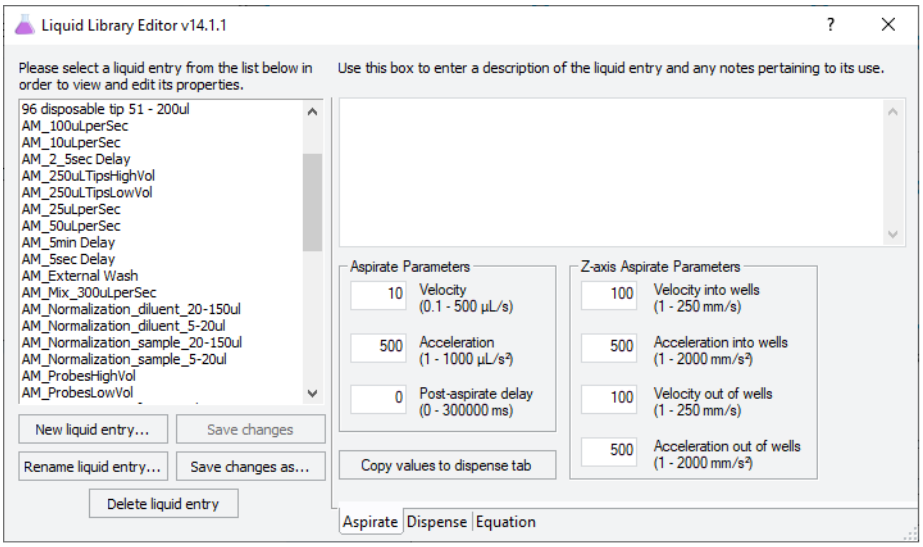
2 Click **Tools > Liquid Library Editor**. The Liquid Library Editor opens.

If the menubar is not visible in the window, right-click the window and select **Menubar** in the shortcut menu that appears.



3 For instructions on how to use the Liquid Library Editor, see the section on specifying pipette speed and accuracy in the *VWorks Automation Control Setup Guide*.

Figure VWorks Liquid Library Editor window



Step 2) Define dilution concentrations

To define the concentration of the serial dilution steps:

In the green boxes in the **Final** column, enter the desired concentration of each serial dilution step.

The method setup tool automatically enters the dilution concentration for dilution 1 when you enter the Final concentration of dilution 1 ("Step 1) Set dilution parameters" on page 662). You enter all the other concentrations in the dilution series directly in each cell or by entering a formula in the cell. You can use the Excel fill series function with formulas to quickly generate a serial dilution.

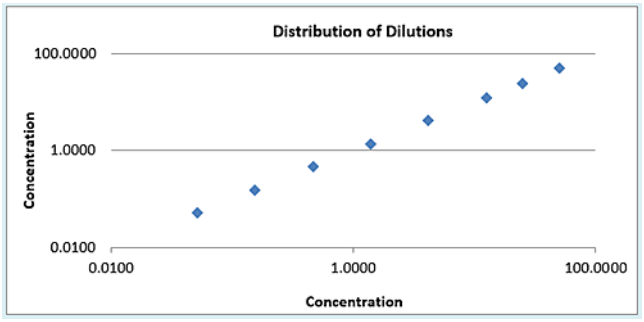
You can generate serial dilutions with 2 to 24 steps. You can also generate blanks by putting zero as a dilution concentration.

Note: If you enter zero as a concentration, an error message appears and says **Negative or zero values cannot be plotted correctly on log charts. Only positive values can be interpreted on a logarithmic scale**. To continue and use the zero, click OK. The graph will not include the zero volume but the method will work.

The Post Run column contains non-editable cells that show the concentration of the serial dilution series after the run is complete.

- If step 1 **Optional volume to be added** is >0, the Post Run column will show the concentration of the serial dilution steps after the serial dilution utility is run but before the optional volume is added. The concentrations entered in the **Final** column represent the concentration of the serial dilution after adding the optional volume.
- If step 1 **Optional volume to be added** is 0, the concentrations entered in the **Final** column will be the same as the those in the Post Run column.

As you define the concentrations, the graph displays the corresponding log-log plot of the dilution series concentrations. You can use the graph to verify that your dilution formula will yield the expected results. The graph can help you visualize the distribution curve and identify gaps between dilution steps.



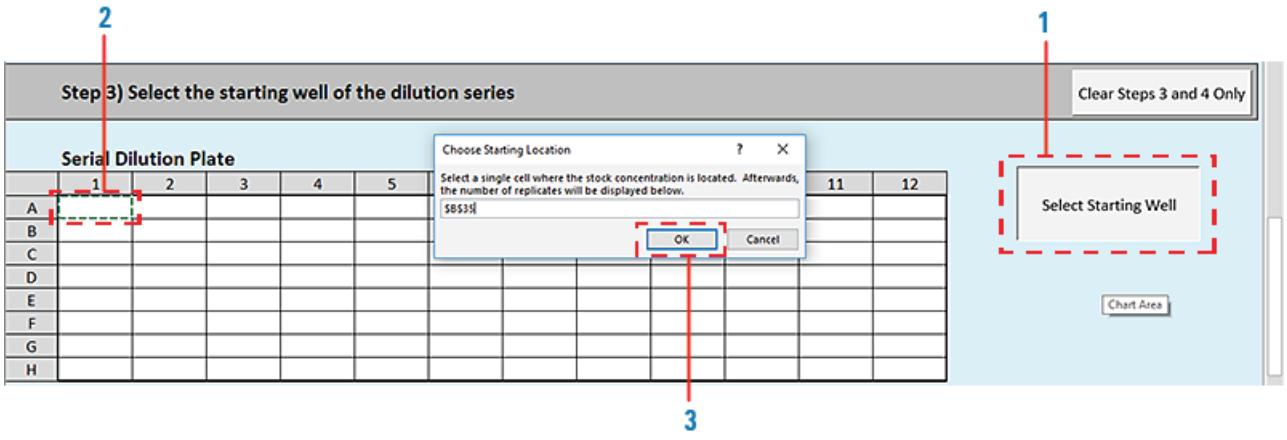
Step 3) Select the starting well of the dilution series

IMPORTANT

If you change anything in step 1 or 2 after starting step 3 or 4, make sure that you click **Clear Steps 3 and 4 Only** and redo steps 3 and 4.

To define the layout of the serial dilution plate:

1 Click **Select Starting Well** (figure, item 1) and then click the corresponding cell in the **Serial Dilution Plate** map where the replicate 1 of dilution 1 will be located (figure, item 2).



2 When the **Choose Starting Location** dialog box opens, displaying a formula, click **OK** (figure, item 3).

The layout for the dilution series appears in the Serial Dilution Plate map, displaying the selected number of replicates in the specified layout (row or column).

Figure Example of a left-to-right layout by row for a dilution series with 4 replicates

	1	2	3	4	5	6	7	8	9	10	11	12
A	12.5000	6.2500	3.1250	1.5625	0.7813	0.3906	0.1953	0.0977	0.0488	0.0244	0.0122	0.0061
B	12.5000	6.2500	3.1250	1.5625	0.7813	0.3906	0.1953	0.0977	0.0488	0.0244	0.0122	0.0061
C	12.5000	6.2500	3.1250	1.5625	0.7813	0.3906	0.1953	0.0977	0.0488	0.0244	0.0122	0.0061
D	12.5000	6.2500	3.1250	1.5625	0.7813	0.3906	0.1953	0.0977	0.0488	0.0244	0.0122	0.0061
E	0.0031	0.0015	0.0008	0.0000								
F	0.0031	0.0015	0.0008	0.0000								
G	0.0031	0.0015	0.0008	0.0000								
H	0.0031	0.0015	0.0008	0.0000								

Figure Example of a top-to-bottom layout by column for a dilution series with 4 replicates

	1	2	3	4	5	6	7	8	9	10	11	12
A	12.5000	12.5000	12.5000	12.5000	0.0488	0.0488	0.0488	0.0488				
B	6.2500	6.2500	6.2500	6.2500	0.0244	0.0244	0.0244	0.0244				
C	3.1250	3.1250	3.1250	3.1250	0.0122	0.0122	0.0122	0.0122				
D	1.5625	1.5625	1.5625	1.5625	0.0061	0.0061	0.0061	0.0061				
E	0.7813	0.7813	0.7813	0.7813	0.0031	0.0031	0.0031	0.0031				
F	0.3906	0.3906	0.3906	0.3906	0.0015	0.0015	0.0015	0.0015				
G	0.1953	0.1953	0.1953	0.1953	0.0008	0.0008	0.0008	0.0008				
H	0.0977	0.0977	0.0977	0.0977	0.0000	0.0000	0.0000	0.0000				

Step 4) Calculate the sample and diluent volumes

To calculate the sample and diluent volumes:

Click **Calculate Volumes**. The tool uses the information provided in the previous steps to calculate the sample and diluent volumes that will be transferred into each well to achieve the specified serial dilution series.

The calculated volumes (µL) appear in the following areas:

- Sample Volume plate map
- Diluent Volume plate map

Sample Volume

	1	2	3	4	5	6	7	8	9	10	11	12
A	80.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
B	80.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
C	80.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
D	80.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
E	35.0	30.0	20.0	0.0								
F	35.0	30.0	20.0	0.0								
G	35.0	30.0	20.0	0.0								
H	35.0	30.0	20.0	0.0								

Calculate Volumes

Diluent Volume

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
B	0.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
C	0.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
D	0.0	40.0	40.0	40.0	40.0	40.0	39.9	39.8	39.7	39.4	38.8	37.5
E	35.0	30.0	20.0	40.0								
F	35.0	30.0	20.0	40.0								
G	35.0	30.0	20.0	40.0								
H	35.0	30.0	20.0	40.0								

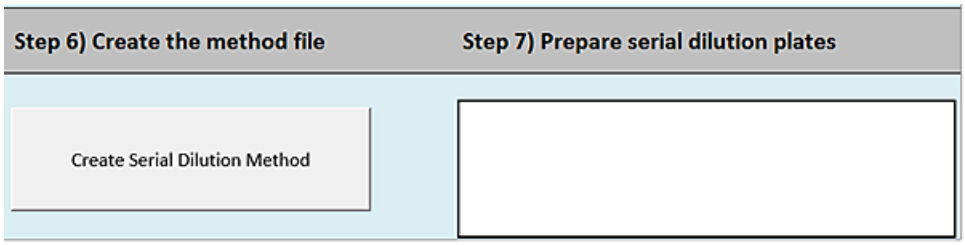
Step 5) Correct these errors

Although you may enter values that are not possible or practical given the selected labware or specified concentrations and volumes, the setup tool will flag such values as errors. You must resolve any errors before you can proceed to the next step in the setup tool.

IMPORTANT

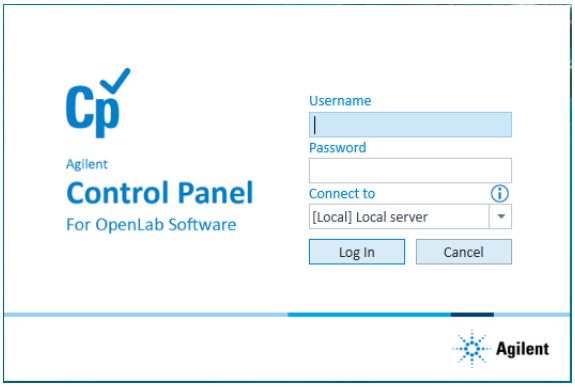
If you change anything in step 1 or 2 at this point, click **Clear Steps 3 and 4 Only** and redo steps 3 and 4.

Step 6) Create the method file

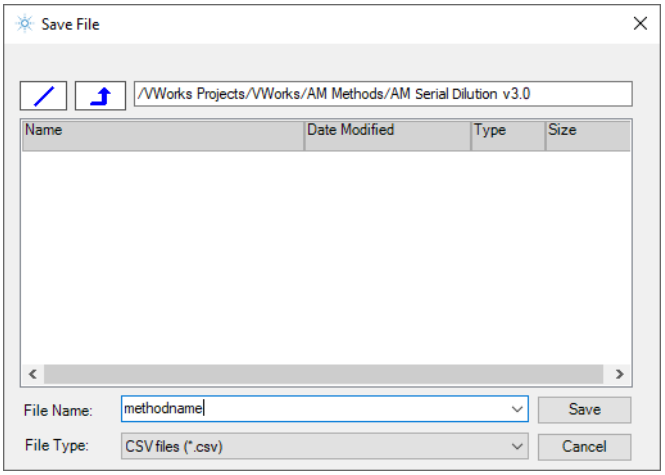


To create the Serial Dilution method file:

1. Click **Create Serial Dilution Method**.
2. In the login window, type your VWorks user name and password, and click **Log In**.



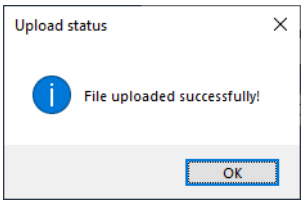
3. In the **Save File** dialog box, specify the file name and the storage location, and then click **Save**.



- VWorks Plus default storage location:
  … /VWorks Projects/VWorks/AM Methods/AM Serial Dilution v3.0
- VWorks Standard default storage location:
  C:\OLSS Projects\VWorks Projects\VWorks\AM Methods\AM Serial Dilution v3.0

Note: The method file is stored as a record of interest (ROI) in an archive (.csv.roiZip). VWorks cannot load files (.roiZip extension) that have been modified or renamed outside of the Protein Sample Prep Workbench or VWorks software.

4 Click **OK** when the uploaded successfully message appears. The Method Setup Tool closes.



Next steps:

If you want to view the method in Microsoft Excel, see "Exporting and importing AssayMAP methods" on page 17

If you are ready to prepare the serial dilution plates, see "Preparing the diluent and samples" on page 672.

Preparing the diluent and samples

Before you start

If you have not already done so, display the method's labware selections and diluent and sample preparation instructions by doing one of the following:

- Open the Serial Dilution utility and select the method. For details, see "Setting up the protocol" on page 675.
- Export the method and view it in Microsoft Excel. For details, see "Exporting and importing AssayMAP methods" on page 17.

CAUTION **Incorrect labware selections can cause a hardware collision, resulting in equipment damage. Ensure that the selections in the method exactly match the physical labware present on the Bravo deck.**

Preparing the diluent reservoir and serial dilution plates

To prepare the diluent reservoir plate:

Follow the method's instructions to prepare the diluent volume (µL) necessary for each dilution series replicate and the well locations where this diluent volume should be placed.

In addition to the diluent volume required to generate the serial dilution curve, some volume overage is required to account for the dead volume in the plate well. For recommended overage volumes and well capacities, see the *Labware Reference Guide* (workbench Literature Library page).

- For 96-well plates, this volume calculation is straightforward in that it is the volume required plus the recommended overage for the well.
- For plates where multiple wells on a 96-well plate map are combined in a common well, the situation can be more complex.
  - If only a single serial-dilution series will draw from the common well (e.g., using an 8-channel reservoir where the serial dilutions go from left to right) the situation is the same as in a 96-well plate where the volume required in the common well is the volume indicated in step 7 plus the required overage for the common well.
  - If more than 1 serial dilution will draw from a common well (e.g., using an 8-channel reservoir where the serial dilutions are going from top to bottom and you have more than one replicate) you would add all the required volumes indicated in step 7 plus the required overage for the common well.

If necessary, use the **Reagent Transfer** utility to transfer the diluent into the appropriate labware type. For instructions, see "Reagent Transfer v3.0 User Guide" on page 525.

To prepare the serial dilution plate:

Follow the method's instructions to prepare the volume (µL) and concentration of the first dilution for each dilution series replicate and the well locations where this starting volume should be placed.

Ensure that the samples in the serial dilution plate match the volume and well positions specified in the method created using the Method Setup Tool.

If necessary, use the **Reformatting** utility to transfer the samples into the appropriate labware type. For instructions, see the Reformatting v3.0 User Guide.

IMPORTANT

To minimize evaporation, fill the labware immediately before run time or keep them covered until you run the protocol.

Running the protocol

The Serial Dilution protocol does the following:

- Washes and dries the syringes.
- Transfers the appropriate volume of diluent and source sample into the designated wells of the serial dilution plate.

Experiment ID and method requirements

Each workbench application and utility has an Experiment Settings section that allows you to select an experiment ID and a method.

- An *experiment ID* is a database record that captures the steps executed and the settings used during each run of an application or utility. Any errors that may have occurred during a run are also recorded.

  To create an experiment ID, you open the Experiments Editor by clicking Experiments Editor in any Workbench app or utility. For details, go to the Literature Library and open *Using the Protein Sample Prep Workbench*. In the browser that opens, click **Using Experiment IDs**.
- A *method* is a comprehensive collection of saved settings for an application or utility, which you can use to run the application or utility.

Experiment IDs and methods are required for compliance-enabled VWorks editions and optional for noncompliance-enabled VWorks editions.

VWorks edition	Experiment ID and method selection
VWorks Plus	Required
VWorks Standard	Optional

Before you start

Ensure that you:

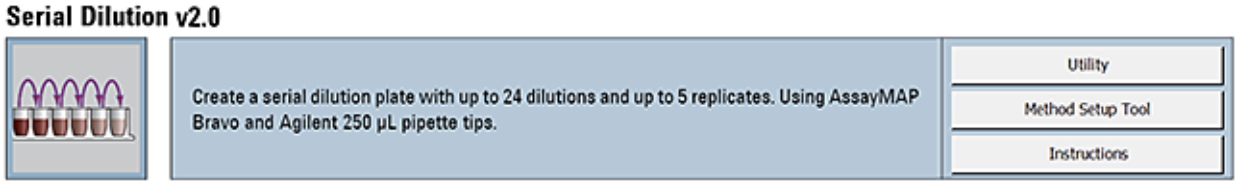
- Prepare the serial dilution setup method. See "Setting up a Serial Dilution method" on page 658.
- Display the method's labware selections and diluent preparation instructions by doing one of the following:
  - Open the Serial Dilution utility and select the method. For details, see "Setting up the protocol" on page 675.
  - Export the method and view it in Microsoft Excel. For details, see "Exporting and importing AssayMAP methods" on page 17.

- Prepare the diluent reservoir and serial dilution plates. See "Preparing the diluent and samples" on page 672.
- If applicable, make sure that you know which experiment ID to use to record the steps executed during the utility and app runs.
- If necessary, run the Startup protocol to initialize the AssayMAP Bravo Platform and prime the wash lines. See the "System Startup/Shutdown v3.0 User Guide" on page 574.

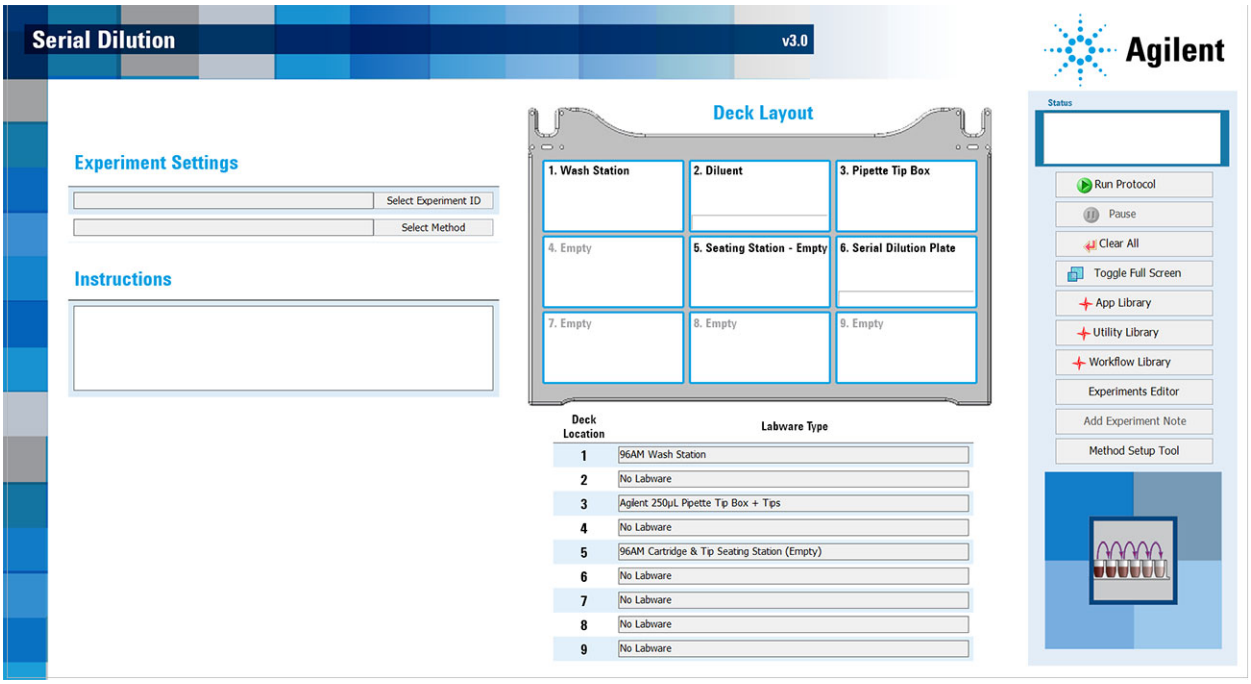
Setting up the protocol

To set up the protocol:

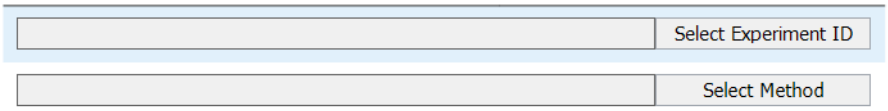
1 Locate the **Serial Dilution** banner in the **Utility Library**, and then click **Utility**.



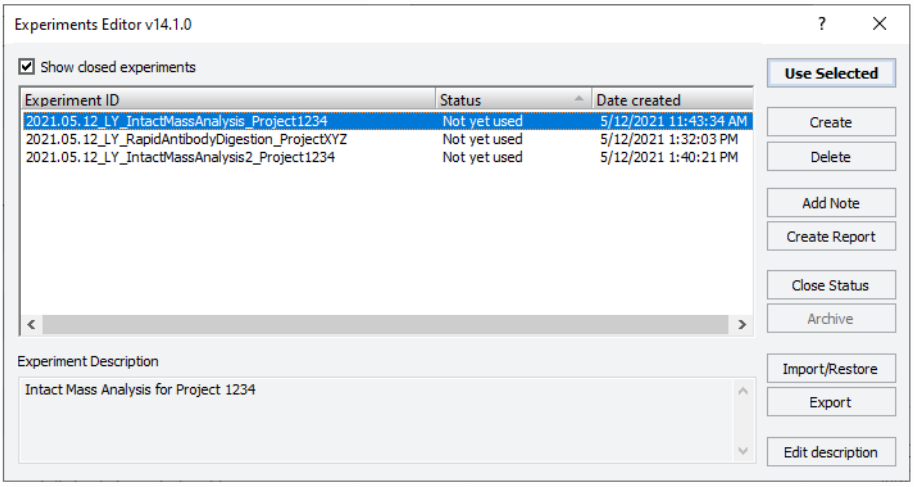
The Serial Dilution utility opens.



2 If applicable, click **Select Experiment ID**.



The Experiments Editor opens.



3 Select the **Experiment ID** that you want to use to record the steps performed during this application run, and then click **Use Selected**.

The Experiments Editor closes.

4 In the form, click **Select Method** to locate and select a method.

In the **Open File** dialog box, select the method, and click **Open**.

The form displays the method labware selections and preparation instructions.

- To run the selected method, go to "Starting the protocol run" on page 676.
- To create or modify a method, see "Setting up a Serial Dilution method" on page 658.

VWorks Plus. Administrator or technician privileges are required to create and modify methods.

About performing a mock run (optional)

If you are unfamiliar with the protocol and would like to see how it operates before running it with valuable samples and reagents, you can perform a mock run. A mock run uses empty or water-filled labware and source bottles.

You prepare for a mock run the same way you would prepare for a real protocol run, except that you use empty labware for a totally dry run or labware containing water for a wet run. You must use pipette tips for the mock run.

Starting the protocol run

WARNING **The probes of the Bravo 96AM Head are sharp and can scratch you if they brush across your hand. A probe scratch can expose you to any contaminants remaining on the probes. Be careful to avoid touching the probes.**

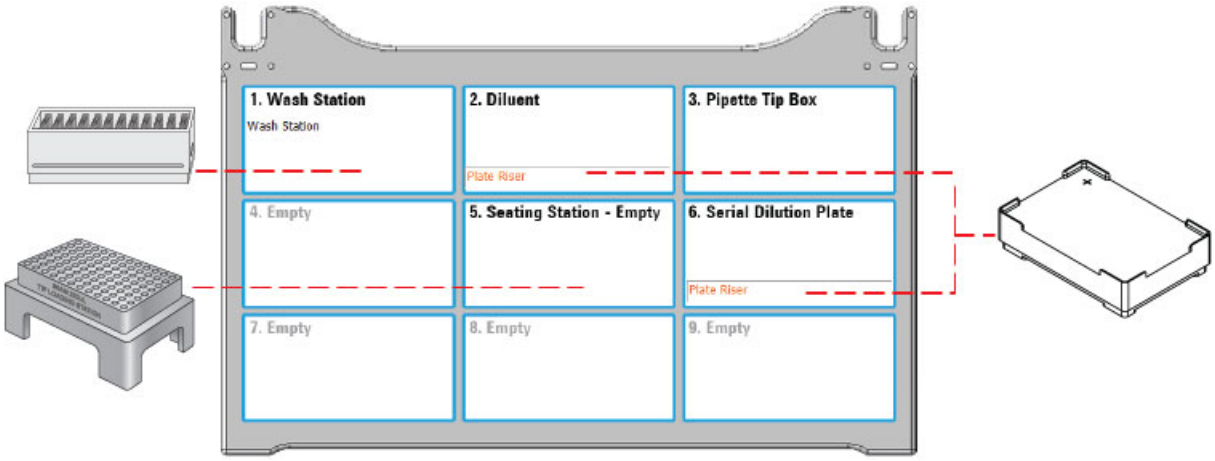
To start the protocol run:

1 Ensure that the accessories are at the assigned deck locations, as shown in the **Deck Layout** image of the form.

- The Bravo Plate Risers are securely in place at deck locations 2 and 6. The diluent reservoir plate is seated on the Bravo Plate Riser at deck location 2. The serial dilution plate is seated on the Bravo Plate Riser at deck location 6.
- The tip box full of fresh pipette tips is at deck location 3 and the empty 96AM Cartridge & Tip Seating Station is at deck location 5.

  Note: A full tip box is required for each run even if the run uses less than 96 pipette tips.

  Note: The protocol will transfer the pipette tips to the seating station.

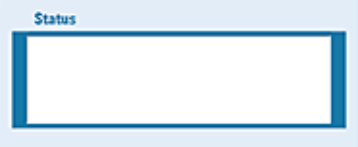


CAUTION **To prevent a potential collision, ensure that no thermal plate insert is on the Peltier Thermal Station installed at deck location 4.**

CAUTION **Incorrect labware selections and improperly seated labware can cause hardware collisions, resulting in equipment damage. Ensure that the selections in the Labware Table exactly match the physical labware present on the Bravo deck. Also ensure that all labware are properly seated within the alignment features of their respective platepads.**

2 Click Run Protocol to start the run.

To monitor the progress of the run, check the **Status** box.



Note: The run time varies depending on the method. The software updates the run time throughout the course of the run, and the completion time estimate becomes more and more accurate as the run progresses.

After the Serial Dilution run. You can run the Reagent Transfer utility to add the Optional volume to be added to the serial dilution plate, if specified in your method.

WARNING **To stop a run in an emergency, use the hardware Emergency Stop button.**

To pause the run, click **Pause**. The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens. For details, see "Emergency stops and pauses" on page 683.

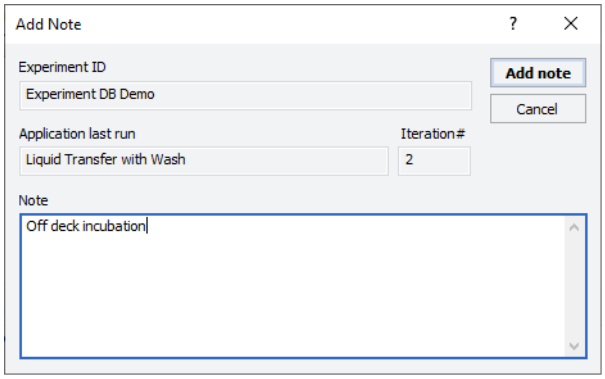
To troubleshoot errors, see the *Error Recovery Guide* and the *Bravo Platform User Guide* in the Literature Library page of the Protein Sample Prep Workbench.

Adding an experiment ID note after the run

After the protocol run ends or during a pause, you can add a note to the experiment ID. For example, a note can describe any observations during the run or any offline steps that are being executed. The notes that you add will appear in any reports generated for the experiment ID.

To add a note to an open experiment ID:

1 While the experiment ID is still selected in the Experiment Settings area, click Add Experiment Note. The Add Note dialog box opens.



2 In the **Note** area, type the note, and then click **OK**.

For detailed instructions on working with Experiment IDs, see "Using Experiment IDs" on page 23.

Cleaning up

Note: Pipette tips that remain in the seating station after the run completion are unused, and they can be reclaimed for future use.

To clean up after a run:

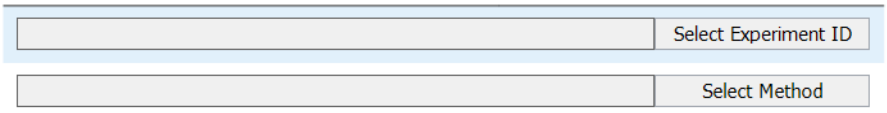
1 Remove used labware from the deck.

2 Discard leftover reagents appropriately.

3 *Optional*. Conduct stringent washing of the syringes:

   a Open the **Syringe Wash** utility  .

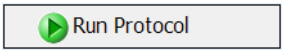
   b If applicable, click **Select Experiment ID** to open the Experiments Editor.



c In the **Experiments Editor**, select the **Experiment ID** that you want to use to capture the steps performed during this utility run, and then click **Use Selected**.

d Click **Select Method** to select and load the method for this utility.

e Confirm that the labware and accessories on the AssayMAP Bravo deck match the display in the **Deck Layout** area of the form.

f Click 

to start the run.

WARNING **Make sure you discard the chemical waste and used labware according to your lab's waste disposal procedures and in compliance with all local, state, and federal safety regulations.**

To shut down at the end of the day:

Run the System Shutdown utility. See "System Startup/Shutdown v3.0 User Guide" on page 574.

Assay development guidelines

This topic provides guidelines for adjusting the protocol, and notes about the application.

Note: For maximum well capacities and overage recommendations in the serial dilution plates, see the *Labware Reference Guide* in the Literature Library of the Protein Sample Prep Workbench.

Automation movements and stepwise guidelines

This section describes the basic automation movements of the AssayMAP Bravo Platform during the Serial Dilution run.

Protocol step	Head moves to deck location...	Action
1. Syringe Wash	1	Performs 1 external syringe wash at the wash station.

Protocol step	Head moves to deck location…	Action
2. Syringe Drying	1	Performs 4 syringe aspirate-and-dispense cycles above the wash station to cycle air in and out of the syringes. The syringes move over the chimneys after each cycle to remove any droplets that were pushed out of the syringes during the cycle.
3. Initial Tip Transfer	3	Presses on all the 250-µL pipette tips from the tip box. *Note:* The tip box must be full even when using fewer than 96 pipette tips because the tip-seating force has been set assuming that a full rack of pipette tips are being seated.
	5	Ejects the pipette tips into the seating station.
4. Offset Head Tip Pickup	5	Positions the head at an offset from the seating station to pick up between 1 to 5 pipette tips, depending on the number of replicates, from the next available column or row of pipette tips. • Picks up tips in row format for top-to-bottom or bottom-to-top dilution series. • Picks up tips in column format for left-to-right or right-to-left dilution.
5. Diluent Transfer	2	Moves to the diluent reservoir. • If a blowout volume is specified, aspirates an air gap. This volume is used to ensure that no liquid remains in the pipette tip after the contents of the tip are dispensed. A small blowout volume can help improve precision and accuracy. The magnitude of this effect is usually small for aqueous solutions but can be significant with viscous solutions. Default: 5 µL Range: 0–50 µL *Note:* The size of the air gap reduces the possible pipetting volume per pipetting cycle. Larger air gaps have diminishing returns in terms of improved accuracy and precision. The relationship between the size of the air gap and the precision and accuracy is highly dependent on the solution being moved. Agilent recommends starting with the default value to determine if any change is required. • If specified, prewets the pipette tips in the designated source wells. Prewetting pipette tips is best practice for good pipetting accuracy and precision. Default: Yes Options: Yes, No • Aspirates diluent into the pipette tips.
	6	• Dispenses the diluent into the designated wells in the serial dilution plate. • If a blowout volume is specified, the head raises to near the top of the well, and the specified volume of air is dispensed followed by a tip touch on the east and west sides of the well. This aspirate-and-dispense process repeats using the same pipette tips until all the wells in the serial dilution plate have the specified amount of diluent. If a blowout is specified, an air gap is aspirated at the start of each aspirate task and a blowout and tip touch occur after each dispense task.

Protocol step	Head moves to deck location...	Action
6. Change Tips	3	Ejects the used pipette tips into the tip box.
	5	Picks up between 1 to 5 pipette tips, depending on the number of replicates, from the next available column or row of pipette tips in the seating station. • Picks up tips in row format for top-to-bottom or bottom-to-top dilution series. • Picks up tips in column format for left-to-right or right-to-left dilution.
7. Serial Dilution Transfer	6	Moves to the lowest concentration dilution in the serial dilution plate. • If a blowout volume is specified, aspirates an air gap. For details, see protocol step 4, above. • If specified, prewets the pipette tips in the designated wells. • Aspirates the sample into the pipette tips, and then dispenses the sample into the next set of dilution wells in the serial dilution plate. • If specified, mixes the well contents for the set number of cycles. The volume used for the mixing process is automatically calculated using the following formula. – If (x - 50 µL) > Tip Capacity, then Mixing Volume = Tip Capacity – If 50 µL ≤ *x* ≤ Syringe/Tip Capacity, then Mixing Volume = 0.75*x* – If *x* < 50 µL, then Mixing Volume = 0.5*x* where, *x* is the final volume in the destination wells, and maximum 250 µL Tip Capacity = 140 µL Default: 10 Range: 0–100 *Note:* – Generally, aqueous solutions are well mixed by 10 cycles if the maximum intermediate volume in the serial dilution (see step 4 in the setup tool) is > 200 µL. The intermediate serial dilution volumes are always higher than the final volume. More viscus and larger volume solutions require more mix cycles, which must be determined empirically. – The more mix cycles you use, the longer the run time. • If a blowout volume is specified, the head raises to near the top of the well, and the specified volume of air is dispensed followed by a tip touch on the east and west sides of the well.
8. Optional Change Tips	3	If the change tips option is specified, ejects the used pipette tips into the tip box. If the change tips option is not specified, this step is skipped and the same set of pipette tips are used for all the serial dilution transfers.
	5	Picks up between 1 to 5 pipette tips, depending on the number of replicates, from the next available column or row of pipette tips in the seating station. • Picks up tips in row format for top-to-bottom or bottom-to-top dilution series. • Picks up tips in column format for left-to-right or right-to-left dilution.

Protocol step	Head moves to deck location...	Action
9. Additional Serial Dilution Transfers	6	Moves to the lowest concentration wells of the dilution series in the serial dilution plate. • If a blowout volume is specified, aspirates an air gap. • If specified, prewets the pipette tips with the sample. • Aspirates the sample into the pipette tips, and then dispenses the sample into the next set of dilution wells in the serial dilution plate. • If specified, mixes the well contents for the set number of cycles. • If specified, performs a blowout, followed by a tip touch on the east and west sides of the wells. • Repeats steps 7 and 8 until the serial dilution series is complete.
10. Final Tip Ejection	3	Ejects the used pipette tips into the tip box.
11. Park Head	1	Moves to a safe height above the wash station.

Emergency stops and pauses

Stopping in an emergency

To stop in an emergency:

Press the red button on the emergency-stop pendant. The Bravo head stops immediately.

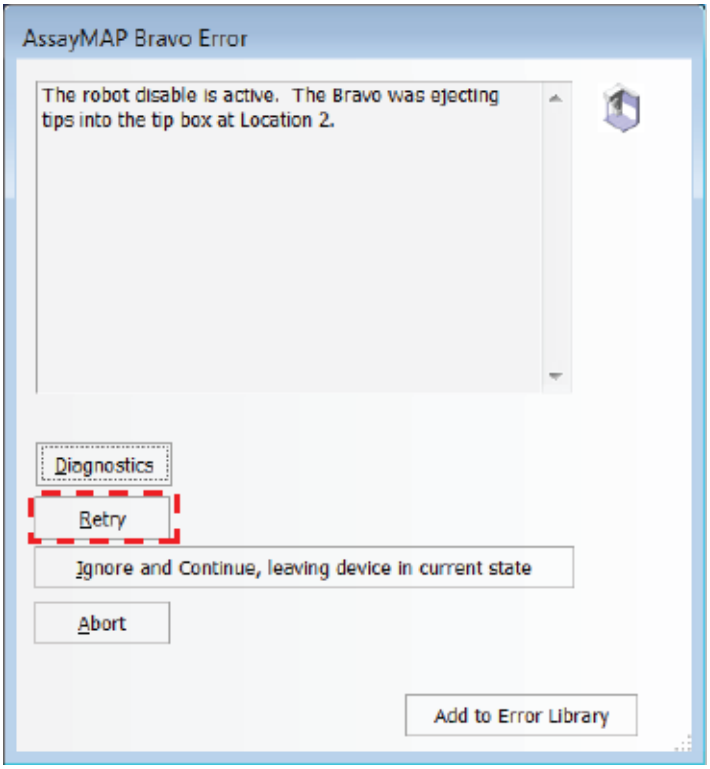
The **robot disable is active** error message opens.

To restore the** Bravo **device after an emergency stop:

1. At the emergency-stop pendant, turn the red button clockwise. The spring-loaded button pops up.

2. *If applicable*. Remove any object that is interrupting the Light Curtain.
3. In the **AssayMAP** Bravo **Error** dialog box, click **Retry**.

   In most cases, the AssayMAP Bravo Platform will be able to resume the run where it left off.



Pausing a run

To pause and then continue a run:

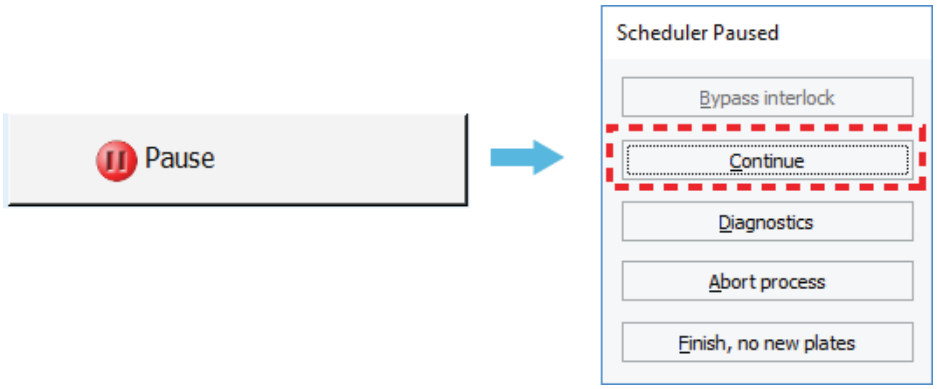
1. In the workbench application or utility control panel, click **Pause**.

   The task currently in progress finishes before the protocol pauses. The Scheduler Paused dialog box opens.

2. While the Bravo device is idle, make the necessary changes to your run, for example, adjusting a labware position or volume.

3. To resume the run, click **Continue** in the **Scheduler Paused** dialog box.

   To cancel the run, click **Abort process** in the **Scheduler Paused** dialog box.



In This Book

This guide includes the user guides for all the applications and utilities in the AssayMAP Protein Sample Prep Workbench.

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