



Avida DNA Reagent Kits

Automated using Agilent NGS Workstation Option B

Library preparation, target capture, and indexing for targeted sequencing on the Illumina platform

Protocol

Version A1, April 2024

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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.

In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end sequencing libraries using Avida DNA Reagent Kits. Sample processing steps are automated using the Agilent NGS Workstation Option B.

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

2 Using the Agilent NGS Workstation with the Avida DNA Workflow

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the Avida DNA library preparation and target capture workflow, and considerations for designing Avida experiments for automated processing using the Agilent NGS Workstation.

3 gDNA Fragmentation

This chapter describes the steps to fragment gDNA samples using mechanical shearing. gDNA samples must be fragmented prior to starting the Avida DNA workflow.

4 Library Preparation and Hybridization

This chapter describes the steps for automated library preparation and hybridization plate setup with the Avida DNA panel. Libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

5 Capture and Washing

This chapter describes the steps for automated target capture and washing using Capture Beads coated with Streptavidin.

6 Indexing PCR

This chapter describes the steps for automated plate setup for the indexing PCR reactions.

7 Library Purification, Quality Assessment, and Sample Pooling

This chapter describes the steps for automated library purification, quality analysis plate setup, and optional sample pooling.

8 Sequencing and NGS Analysis

This chapter contains guidance on library sequencing and analysis. Refer to your specific Illumina sequencer's user guide for specific instructions on how to perform sequencing.

9 Reference

This section contains reference information, including Reagent Kit contents, index primer pair information, and troubleshooting tips.

1	Before You Begin	
	Introduction	7
	Avida Target Enrichment Technology	7
	Procedural and Safety Notes	8
	Important practices for preventing contamination	8
	Safety notes	8
	Procedural notes	8
	Materials Provided with Avida DNA Kits and Panels	10
	Online resource for Avida index sequences	10
	Additional Materials Required	11
	Input DNA samples	11
	Additional reagents and equipment required	11
	Equipment required for gDNA fragmentation	13
2	Using the Agilent NGS Workstation with the Avida DNA Workflow	
	About the Agilent NGS Workstation	15
	About the Bravo Platform	15
	VWorks Automation Control Software	18
	Overview of the Avida DNA VWorks Automation Protocols	23
	Experimental Setup Considerations for Automated Runs	24
	Considerations for Experimental Setup	24
	Plate Type Considerations	24
3	gDNA Fragmentation	
	Step 1. Prepare for shearing	27
	Step 2. Shear the gDNA samples	27
4	Library Preparation and Hybridization	
	Step 1. Prepare the NGS Workstation	30
	Step 2. Prepare plates for the DNA samples, Hyb Buffer, and Library Wash Buffer	30
	Step 3. Prepare the End Prep master mix	32
	Step 4. Prepare the Ligation master mix	32
	Step 5. Prepare the Adaptor mix	33
	Step 6. Prepare the Hyb master mix	33
	Step 7. Prepare the Library Prep master mix reagent source plate	34
	Step 8. Load the NGS Workstation	36
	Step 9. Run VWorks runset LibraryPrep_Avida_ILM	37
	Step 10. Perform hybridization	39

5	Capture and Washing	
	Step 1. Prepare the NGS Workstation	41
	Step 2. Prepare the Capture Bead source plate	41
	Step 3. Prepare remaining plates for target capture and washing	44
	Step 4. Load the NGS Workstation	45
	Step 5. Run the target capture and washing runset or protocol	46
6	Indexing PCR	
	Step 1. Prepare the NGS Workstation for the PCR_Avida_ILM protocol	50
	Step 2. Prepare the 2X Amplification Master Mix source plate	50
	Step 3. Prepare the Index Primer Pairs plate	51
	Step 4. Load the NGS Workstation for the PCR_Avida_ILM protocol	52
	Step 5. Run VWorks protocol PCR_Avida_ILM	53
	Step 6. Perform PCR cycling	53
7	Library Purification, Quality Assessment, and Sample Pooling	
	Library Purification	56
	Step 1. Prepare the AMPure XP bead plate	56
	Step 2. Prepare the 1X Low TE Buffer plate	56
	Step 3. Prepare the NGS Workstation	56
	Step 4. Run VWorks protocol AMPureXP_Cleanup	58
	Library Quantity and Quality Assessment	59
	Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape	59
	Option 2: Analysis using an equivalent platform (non-automated)	63
	Sample Pooling for Multiplexed Sequencing (optional)	65
	Option 1: Pool samples at equal volumes (non-automated)	65
	Option 2: Pool samples at varying volumes (automated or non-automated)	65
8	Sequencing and NGS Analysis	
	Step 1. Prepare the sequencing samples	70
	Step 2. Sequence the libraries	70
	Step 3. Process and analyze the reads	71
9	Reference	
	Reagent Kit Contents	74
	Avida Sub-Kit Details	74
	Avida Index Primer Pair Information	75
	Index Primer Pair Strip Tube and Plate Maps	75
	Avida Custom AddOn Panels	77
	Troubleshooting Guide	78

1 Before You Begin

Introduction 7

Procedural and Safety Notes 8

Materials Provided with Avida DNA Kits and Panels 10

Additional Materials Required 11

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

Make sure you have the most current protocol. Go to www.agilent.com and search for G9409-90010.

NOTE

This protocol describes automated Avida DNA sample processing using the Agilent NGS Workstation Option B. For non-automated sample processing procedures see publication G9409-90000.

NOTE

Agilent guarantees performance and provides technical support for the Avida reagents required for this workflow only when used as directed in this Protocol.

Introduction

Genomic alteration is the underlying cause of many diseases including cancer. Targeted sequencing is widely used to analyze disease related to genomic alterations. There is a growing demand in both research and clinical settings for a simple, fast, and ultra-sensitive targeted sequencing solution to assess various ranges of genomic alterations of disease-related regions particularly with a low DNA input. Avida targeted sequencing is a novel technology developed and perfected to retrieve genomic information from virtually any region of interest even for samples with limited quantity. This assay is particularly optimized for circulating cell free DNA (cfDNA), an emerging analyte demonstrating increasing importance in molecular diagnostics. The technology also supports fragmented genomic DNA (gDNA) isolated from fresh/fresh-frozen samples or from FFPE-derived samples. With a simplified workflow and fast hybridization chemistry, Agilent's Avida targeted sequencing technology can provide in-depth DNA analysis in hours for a broad range of genomic alterations with the highest recovery of input DNA.

Avida Target Enrichment Technology

At the core of the Avida target enrichment technology is an interlocked, three-dimensional structure, designed specifically for synergistic, indirect capture of intended DNA targets. In our proprietary design, a DNA scaffold forms with the intended target molecule when – and only when – more than one bridge probe is hybridized to the same target and stabilized by an anchor probe labeled with biotin (see [Figure 1](#)). Compared to traditional hybridization methods that use single and long biotinylated probes, the faster, more efficient Avida hybridization reaction is built on a much shorter, target-specific sequence used in the bridge probes. Consequently, a highly specific capture is achieved when the synergistic hybridization forms this interlocked structure and, in turn, binds to the streptavidin beads.

Avida probe panels are pre-formulated and ready-to-use to deliver consistent and best-in-class performance.

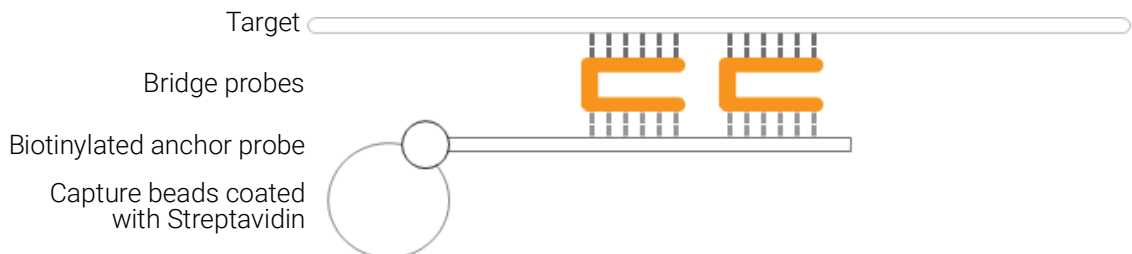


Figure 1 Hybridization of target to biotinylated anchor probe mediated by bridge probes

Procedural and Safety Notes

Important practices for preventing contamination

The Avida DNA technology is highly sensitive, making prevention of contamination of critical importance. Observe the following cautions when performing the workflow.

CAUTION

To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.

CAUTION

To prevent PCR product contamination of samples throughout the workflow:

- Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow. Reagents can be transferred from the pre-PCR area to the post-PCR area, but never from the post-PCR area to the pre-PCR area.
 - Maintain clean work areas. Before each assay, clean the surfaces that pose the highest risk of contamination using a solution of 10% bleach and water, or equivalent. Then, wipe the surfaces with water to remove bleach residue.
 - Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
 - Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
-

Safety notes

WARNING

Wear appropriate personal protective equipment (PPE) when working in the laboratory.

In general, follow Biosafety Level 1 (BSL 1) safety rules.

Procedural notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Workstation to allow rapid and efficient plate transfer.
- Some protocol steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer included with the Agilent NGS Workstation, and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the Agilent NGS Workstation.

Before You Begin

Procedural notes

- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- Prepare and load the Agilent NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the workstation's Labware MiniHub, always place plates in the orientation shown in [Figure 4](#) on page 36.
- Reagents requiring -20°C storage are temperature sensitive and appropriate care should be taken during storage and handling. Avoid extensive freeze-thaw cycles. Make sure all reagents previously stored at -20°C are completely thawed before use.
- Possible stopping points, where samples may be stored at 4°C or -20°C , are marked in the protocol.

Materials Provided with Avida DNA Kits and Panels

Table 1 lists the Avida DNA reagent kits and the sub-kit boxes provided with each kit. The 96-reaction kits provide sufficient reagents for 6 assays with 16 samples.

Table 2 lists the Avida panels compatible with this Protocol.

Table 1 Avida DNA reagent kits

Avida DNA reagent kits	Sub-kit boxes	Storage Temperature
P/N G9418A Avida DNA reagent kit with 1-96 index primer pairs for ILM, 96 reactions	Avida DNA Reagent Box 1, 96 Reactions	-20°C
	Avida DNA and Duo Reagent Box 2, 96 Reactions	Room temperature
	Avida Beads Box, 96 Reactions	4°C
	Avida Index Primer Pairs 1-96 for ILM	-20°C
P/N G9418B Avida DNA reagent kit with 97-192 index primer pairs for ILM, 96 reactions	Avida DNA Reagent Box 1, 96 Reactions	-20°C
	Avida DNA and Duo Reagent Box 2, 96 Reactions	Room temperature
	Avida Beads Box, 96 Reactions	4°C
	Avida Index Primer Pairs 97-192 for ILM	-20°C

Table 2 Compatible Avida panels

Capture Probes Panel	Size	Design ID	Agilent Part Number
Avida DNA Expanded Cancer Panel	345 kb	D3483241	5280-0048
Avida DNA Focused Cancer Panel	26 kb	D3483231	5280-0051

Online resource for Avida index sequences

Each member of the Avida Index Primer Pairs contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. For complete index sequence information, download the Avida Index Sequence Resource Excel spreadsheet using this link: [Avida Index Resource](#).

Note that this link does not open a website. It automatically downloads the Excel spreadsheet to the default folder for downloaded files saved by your web browser. Locate the file in the folder to open it in Microsoft Excel or another compatible spreadsheet program. You can also find a link to the spreadsheet on the Avida DNA product page on www.agilent.com. The first tab of the spreadsheet provides instructions for use of the spreadsheet contents.

Additional Materials Required

Input DNA samples

The requirements for the DNA samples used as the starting material for the workflow are summarized in [Table 3](#). Input quantity ranges are based on Qubit Assay quantification of the DNA samples. DNA should be stored in 1X low TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) or Qiagen Elution Buffer EB (10 mM Tris-HCl, pH 8.5). DNA needs to be at the appropriate size before you begin library preparation. For gDNA, Agilent recommends using Covaris shearing to achieve average insert sizes of 150–300 base pairs (see [Chapter 3](#), “gDNA Fragmentation” for instructions). Since cfDNA typically has an average size of 170 base pairs, no further fragmentation is required.

Table 3 Input DNA requirements by DNA sample type

Sample type	Input quantity	Fragmentation requirements	Storage buffer
cfDNA	1–100 ng [*]	No fragmentation required as average fragment size is already 170 bp	Low TE buffer or Qiagen Buffer EB
gDNA from fresh or fresh-frozen sample or FFPE-derived sample	10–100 ng [†]	Fragmentation with Covaris shearing to insert sizes of 150–300 bp	Low TE buffer or Qiagen Buffer EB

^{*} Libraries can be generated with inputs of cfDNA as low as 1 ng. For variant detection, increasing the input improves performance.

[†] For FFPE-derived gDNA, use the maximum amount of sample available in this range.

NOTE

The Avida workflow is not compatible with the Seraseq ctDNA Complete Reference Material or the Seraseq ctDNA Reference Material V2 available from SeraCare. Contact Agilent [Technical Support](#) with questions.

Additional reagents and equipment required

See [Table 4](#) through [Table 9](#) for additional reagents and equipment required to complete the workflow.

Table 4 Additional required reagents

Description	Vendor and Part Number
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276, or equivalent
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
Qubit HS dsDNA Assay Kit (optional)	Thermo Fisher Scientific
100 assays	p/n Q32851
500 assays	p/n Q32854
Purification Beads: AMPure XP for PCR purification	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881

Before You Begin

Additional reagents and equipment required

Table 5 Required equipment

Description	Vendor and Part Number
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers <i>Important:</i> Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: <ul style="list-style-type: none"> 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 96 Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 96 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401
Nucleic acid analysis system (instrument and consumables)	Select one system from Table 8 on page 13
Heat blocks or water baths heated to 37°C and 50°C	General laboratory supplier
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Magnet for 1.5-mL tubes – only required if manually preparing the Capture Beads; see page 41	Thermo Fisher Scientific p/n 12321D or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Centrifuge for 96-well plate	General laboratory supplier
Multichannel and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, cold rack, ice bucket, and powder-free gloves	General laboratory supplier

Table 6 Bravo NGS Workstation and Workstation plasticware

Description	Vendor and Part Number
Agilent NGS Workstation Option B Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.0.1366) OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Processing plates, 96-well, full-skirted	96-well Eppendorf twin.tec plates (Eppendorf p/n 951020401) OR 96-well Armadillo plates (Thermo Fisher Scientific p/n AB2396)
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height – used when workstation setup calls for Agilent Deep Well Reservoir	Agilent p/n 201244-100

Table 6 Bravo NGS Workstation and Workstation plasticware (continued)

Description	Vendor and Part Number
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when workstation setup calls for Agilent Deep Well Plate or Agilent DW Plate	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when workstation setup calls for Waste Plate (Agilent 2 mL Square Well)	Agilent p/n 201240-100

Table 7 Recommended supplies for decontaminating the NGS Workstation surfaces

Description	Vendor
Dilute bleach (10%) wipes	Hype-Wipe Bleach Towelettes, or equivalent
Isopropanol (70%) wipes	VWR Pre-Moistened Clean Wipes, or equivalent

Table 8 Nucleic acid analysis platform options – select one

Analysis System	Vendor and Part Number Information
Agilent 4200/4150 TapeStation Instrument Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps D1000 ScreenTape D1000 Reagents	Agilent p/n G2991AA/G2992AA p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5582 p/n 5067-5583
Agilent 5200/5300/5400 Fragment Analyzer Instrument Consumables: NGS Fragment Kit (1-6000 bp)	Agilent p/n M5310AA/M5311AA/M5312AA p/n DNF-473-0500

Equipment required for gDNA fragmentation

Table 9 lists information on equipment for fragmentation of gDNA samples using mechanical shearing. Sample fragmentation is required for gDNA samples but not for cfDNA samples.

Table 9 Equipment for mechanical shearing of gDNA samples

Description	Vendor and Part Number	Usage Notes
Covaris Sample Preparation System	Covaris model E220	Additional Covaris instrument models and sample holders may be used after optimization of shearing conditions.
Covaris microTUBE sample holders	Covaris p/n 520045	

2 Using the Agilent NGS Workstation with the Avida DNA Workflow

- About the Agilent NGS Workstation [15](#)
- Overview of the Avida DNA VWorks Automation Protocols [23](#)
- Experimental Setup Considerations for Automated Runs [24](#)

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the Avida DNA library preparation and target capture workflow, and considerations for designing Avida experiments for automated processing using the Agilent NGS Workstation.

About the Agilent NGS Workstation

CAUTION

Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices included with the workstation. Refer to the user guides listed in [Table 10](#).

Review the user guides listed in [Table 10](#) (available at www.agilent.com) to become familiar with the general features and operation of the Agilent NGS Workstation Option B components. Instructions for using the Bravo platform and other workstation components specifically for the Avida DNA workflow are detailed in this user guide.

Table 10 Agilent NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (formerly G5562-90000)
VWorks Software	G5415-90068
BenchCel Microplate Handler	G5580-90000
Labware MiniHub	G5584-90001
PlateLoc Thermal Microplate Sealer	G5585-90010

About the Bravo Platform

The Bravo platform is a versatile liquid handler with a nine plate-location platform deck, suitable for handling 96-well, 384-well, and 1536-well plates. The Bravo platform is controlled by the VWorks Automation Control software. Fitted with a choice of three interchangeable disposable-tip pipette heads, it accurately dispenses fluids from 0.3 μ L to 250 μ L.

Bravo Platform Deck

The protocols in the following sections include instructions for placing plates and reagent reservoirs on specific Bravo deck locations. Use [Figure 2](#) to familiarize yourself with the location numbering convention on the Bravo platform deck.

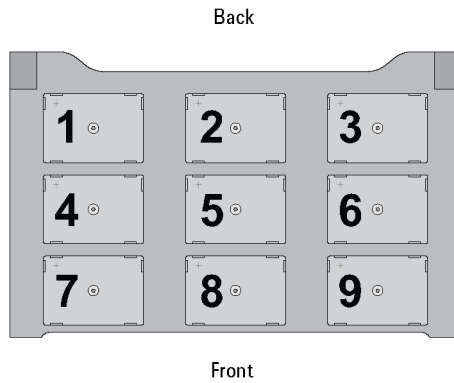


Figure 2 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

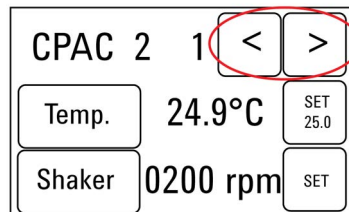
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the run.

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See [Table 11](#) for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

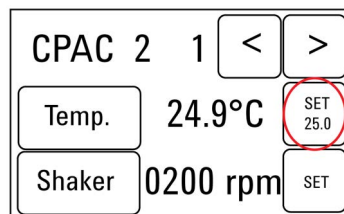
Table 11 Inheco Multi TEC Control touchscreen designations

Bravo Deck Position	Designation on Inheco Multi TEC Control Screen
4	CPAC 2 1
6	CPAC 2 2

- Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).

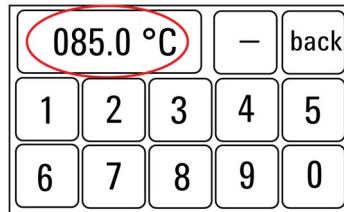


- To set the temperature of the selected block, press the SET button.

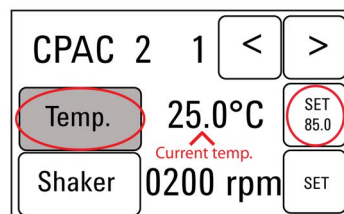


Using the Agilent NGS Workstation with the Avida DNA Workflow About the Bravo Platform

- Using the numeral pad, enter the desired temperature. The entered temperature appears in the top, left rectangle. Once the correct temperature is displayed, press the rectangle to enter the temperature.



- Press the Temp button until the new temperature is displayed on the SET button and until the Temp button is darkened, indicating that the selected heat block is heating or cooling to the new temperature setting. The current temperature of the block is indicated in the center of the display.



Setting the Temperature of Bravo Deck Position 9 Using the ThermoCube Device

Bravo deck position 9 is equipped with a ThermoCube thermoelectric temperature control system, used to incubate components at a defined temperature during the run. During protocols that require temperature control at position 9, you will be instructed to start and set the temperature of the ThermoCube device before starting the run.

ThermoCube temperature settings are modified using the control panel (LCD display screen and four input buttons) on the front panel of the device using the following steps.

- Turn on the ThermoCube and wait for the LCD screen to display TEMP.
- Press the UP or DOWN button to change SET TEMP 1 to the required set point.
- Press the START button.

The ThermoCube then initiates temperature control of Bravo deck position 9 at the displayed set point.

VWorks Automation Control Software

VWorks software, included with your Agilent NGS Workstation, allows you to control the robot and integrated devices using a PC. The Agilent NGS Workstation is preloaded with VWorks software containing all of the necessary system liquid handling protocols for the Avida DNA workflow. General instructions for starting up the VWorks software and the included protocols is provided below. Each time a specific VWorks protocol is used in the Avida DNA procedure, any settings required for that protocol are included in the relevant section of this manual.

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.0.1366

If you have questions about VWorks version compatibility, please contact service.automation@agilent.com.

Logging in to the VWorks software

- 1 Double-click the VWorks icon for the Avida DNA VWorks Form shortcut on the Windows desktop to start the VWorks software.
- 2 If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
- 3 In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

VWorks protocol and runset files

VWorks software uses two file types for automation runs, *.pro* (protocol) files and *.rst* (runset) files. Runset files are used for automated procedures in which the workstation uses more than one automation protocol during the run.

Using the Avida DNA Form to setup and start a run

Use the Avida DNA VWorks form, shown below, to set up and start each automation protocol or runset.

VWorks | Avida DNA on Bravo NGS Option B
Bravo Automated Liquid Handling Protocol of Avida Targeted DNA-Seq for fragmented gDNA or cfDNA

Protocol Parameters

- Select protocol to execute
- Select labware for thermal cycling
- Select number of columns of samples to process
- Check workstation setup
- Load labware and reagents according to Bravo Deck Setup

Select Aliquot Input File

Options

References

Testing Only
 Reduce Incubation Times and Mix Cycles
 DUO Hyb (beta)

NGS Workstation B Setup

Run Status

Protocol Status

Executed Protocol

BenchCel 4R

Stacker 1	Stacker 2	Stacker 3	Stacker 4

MiniHub

	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5				
Shelf 4				
Shelf 3				
Shelf 2				
Shelf 1				

- 1 Open the form using the shortcut (shown below) on your desktop, or by opening the file **Avida_DNA_vB1.x.x.x.VWForm** in the directory **C:\VWorks Workspace\NGS Option B\Avida_DNA_vB1.x.x.x\Forms** (where x.x.x is the version number).



- 2 Use the form drop-down menus to select the appropriate Avida workflow step and number of columns of samples for the run.
- 3 Once all run parameters have been specified on the form, click **Display Initial Setup**.

Display Initial Setup

- 4 The NGS Workstation B Setup region of the form will then display the required placement of reaction components and labware in the NGS Workstation for the specified run parameters.

VWorks | Avida DNA on Bravo NGS Option B
Bravo Automated Liquid Handling Protocol of Avida Targeted DNA-Seq for fragmented gDNA or cDNA

Protocol Parameters

- Select protocol to execute
LibraryPrep_Avida_ILM_v.B1.2.1.2.rst
- Select labware for thermal cycling
96 Agilent Sens-skirted PCR in Red Alum Insert
Processing Plate
96 Eppendorf Twin.tec PCR
- Select number of columns of samples to process
3
- Check workstation setup
Display Initial Setup | Clear Workstation Setup
- Load labware and reagents according to Bravo Deck Setup
Run Selected Protocol
Reset Form to Defaults | Pause

Select Aliquot Input File

Options
Full Screen | Initialize All Devices

References
Master Mix Tables | Gantt Chart

Testing Only
 Reduce Incubation Times and Mix Cycles
 DUO Hyb (beta)

NGS Workstation B Setup

Bravo Deck Setup

1. Waste Plate (Agilent 2mL Square Well)

2.

3.

4. Peltier 67C

5. Shaker
Empty 96 Eppendorf Twin.tec PCR Plate for Library Binding Beads

6. Peltier 20C

7. Magnet
Input DNA in 96 Eppendorf Twin.tec PCR Plate

8.

9. Chiller 0C

Run Status

Elapsed Time: 00:00:00

Protocol Status
Setup for LibraryPrep_Avida_ILM_v.B1.2.1.2.rst

Executed Protocol

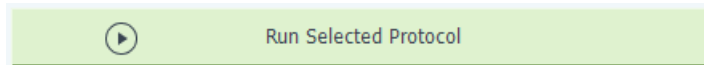
BenchCel 4R

Stacker 1	Stacker 2	Stacker 3	Stacker 4
2 Tip Boxes	Empty	Empty	Empty

MiniHub

	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5	Empty 96 Eppendorf Twin.tec PCR Plate for End Prep Mix			
Shelf 4	Empty 96 Eppendorf Twin.tec PCR Plate for Adaptors			
Shelf 3	Empty 96 Eppendorf Twin.tec PCR Plate for Ligation Mix			
Shelf 2	New Tip Box	30uL Hyb Buffer in 96 Eppendorf Twin.tec PCR Plate		
Shelf 1	Empty Tip Box	Library Wash Buffer in Agilent Deep Well 1mL Plate		Empty Tip Box

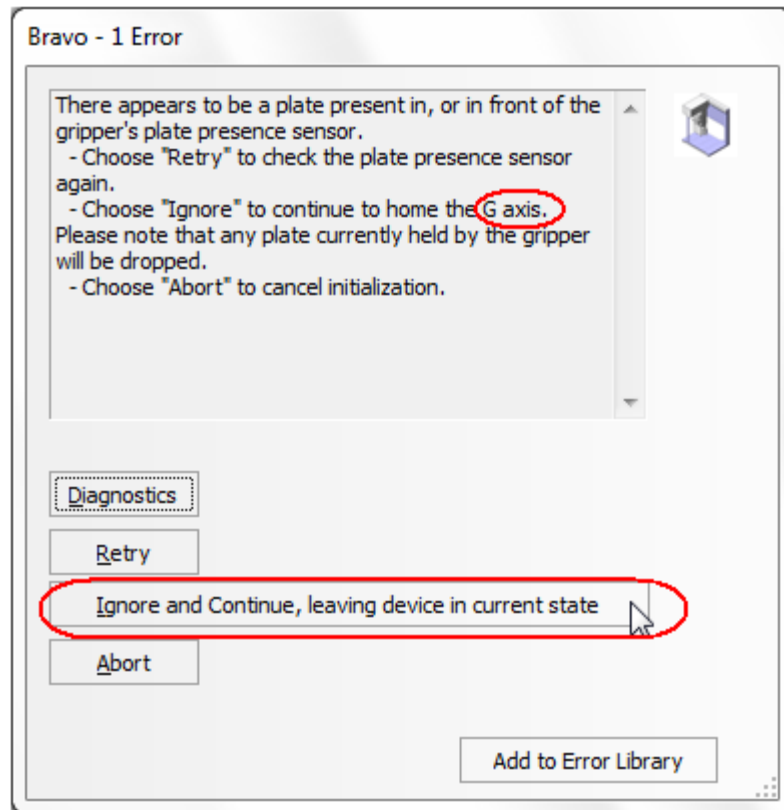
5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.



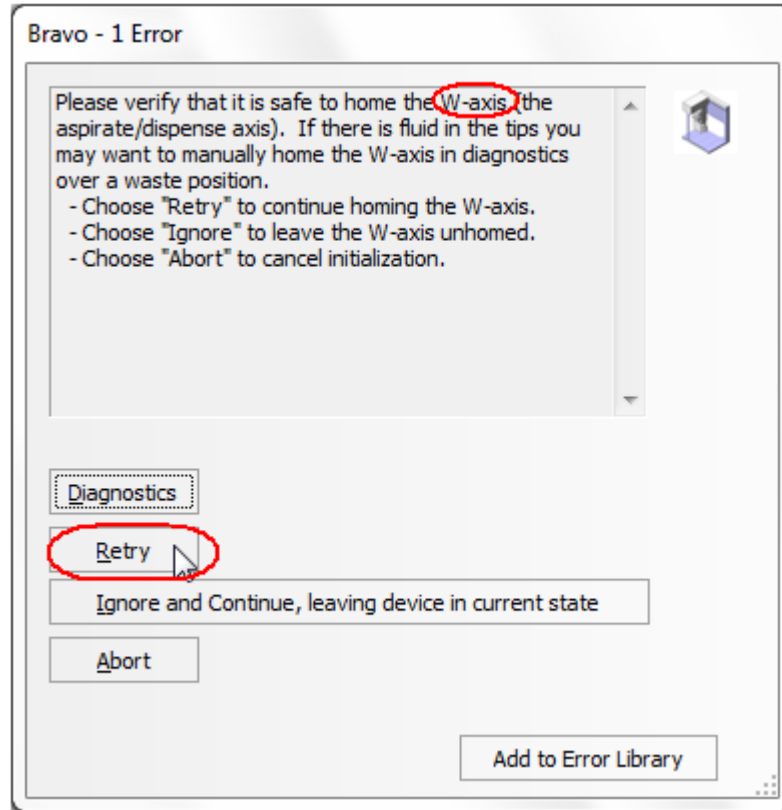
Error messages encountered at start of run

After starting the run, you may see the error messages displayed below. When encountered, make the indicated selections and proceed with the run. Encountering either or both of these error messages is not indicative of a problem with the NGS workstation or your run setup.

- 1 If you encounter the G-axis error message shown below, select **Ignore and Continue**, leaving device in current state.



- 2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

VWorks software may be run in simulation mode, during which commands entered on screen are not completed by the NGS workstation. If workstation devices do not respond when you start a run, verify the simulation mode status in VWorks using the following steps.

- 1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking **View > Control Toolbar**).



- 2 If the indicator displays **Simulation is on**, click the status indicator button to turn off the simulation mode.

NOTE

If you cannot see the toolbar above the VWorks form, click the **Full Screen** button to exit full screen mode. If the toolbar is still not visible, right-click on the form and then select **Control Toolbar** from the menu.

Overview of the Avida DNA VWorks Automation Protocols

See [Table 12](#) for a summary of the VWorks protocols and runsets used during the workflow.

Table 12 Overview of VWorks protocols and runsets

Workflow Step	Substep	VWorks Protocol	Approximate Time*
Library Preparation and Hybridization Set Up	Prepare duplex, molecular-barcoded DNA libraries. Set up hybridization reactions to be run on the thermal cycler.	LibraryPrep_Avida_ILM	3 hours (+1 hour for hybridization)
Target Capture and Wash	Capture and wash DNA hybrids	TargetCapture_Avida (includes preparation of the Capture Beads) -- OR -- Capture&Wash_Avida (uses manually prepared Capture Beads)	1 hour
Indexing PCR	Amplify target-enriched libraries with index primer pairs	PCR_Avida_ILM	15 minutes (+0.5–1 hour for PCR)
Library Purification	Purify enriched, amplified libraries using AMPure XP beads	AMPureXP_Cleanup (Case Post-Capture PCR)	35 minutes
Quality Assessment	Analyze final libraries using Agilent TapeStation platform	TS_D1000	10 minutes (+ up to 1.5 hours for TapeStation assay)
Sample Pooling (optional)	Pool indexed DNA libraries	Aliquot_Captures	up to 1 hour

* Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol.

Experimental Setup Considerations for Automated Runs

Considerations for Experimental Setup

- Automated runs may include 1, 2, 3, 4, 6, or 12 columns (equivalent to 8, 16, 24, 32, 48, or 96 wells) of DNA samples to be enriched for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Table 13 Columns to Samples Equivalency

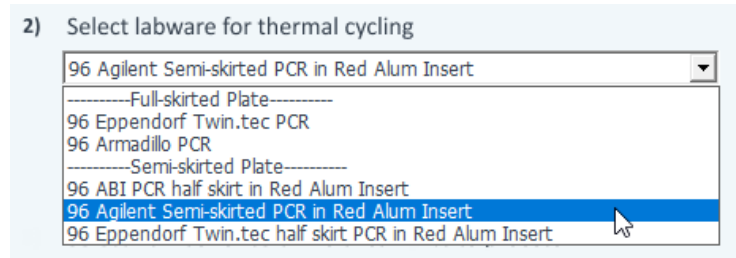
Number of Columns Processed	Total Number of Samples Processed
1	8
2	16
3	24
4	32
6	48
12	96

- The number of columns or samples that may be processed using the supplied reagents (see [Table 1](#)) will depend on the experimental design. For greatest efficiency of reagent use, plan experiments using at least 3 columns per run. Each 96-reaction kit contains sufficient reagents for 96 reactions configured as 4 runs of 3 columns of samples per run.
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. The DNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.

Plate Type Considerations

PCR plate types

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps, you must specify on the VWorks Form which PCR plate type will be used to allow correct configuration of the liquid handling components. Before you begin the automation protocol, make sure that you are using a supported PCR plate type. The PCR plate type to be used in the protocol is specified using the menu below. Vendor and part number information is provided for the supported plate types in [Table 14](#).



CAUTION

The plates listed in [Table 14](#) are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

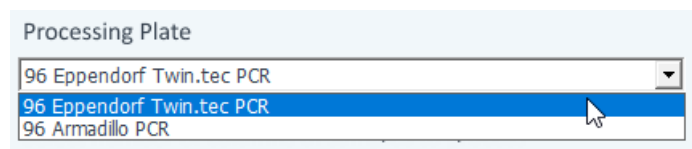
Do not use PCR plates that are not listed in [Table 14](#), even if they are compatible with your chosen thermal cycler.

Table 14 Ordering information for supported PCR plates

Description in VWorks menu	Vendor and part number
-----Full-skirted Plate-----	
96 Eppendorf Twin.tec PCR	Eppendorf p/n 951020401
96 Armadillo PCR	Thermo Fisher Scientific p/n AB2396
-----Semi-skirted Plate-----	
96 ABI PCR half-skirt (MicroAmp Optical plates)	Thermo Fisher Scientific p/n N8010560
96 Agilent Semi-skirted PCR	Agilent p/n 401334
96 Eppendorf Twin.tec half-skirt PCR	Eppendorf p/n 951020303

Processing plate types

Many of the automation protocols use a 96-well processing plate for reagent storage and mixing. The Avida DNA VWorks form allows you to select between two different processing plates, as shown in the menu below. Before running a protocol, verify that the correct processing plate is selected in the menu. Use the same type of processing plate for all protocols and runs in the same run.



The processing plate is either a 96-well Eppendorf twin.tec PCR plate (Eppendorf p/n 951020401) or a 96-well Armadillo PCR plate (Thermo Fisher Scientific p/n AB2396). Do not substitute another plate type.

3 gDNA Fragmentation

Step 1. Prepare for shearing [27](#)

Step 2. Shear the gDNA samples [27](#)

This chapter describes the steps to fragment gDNA samples using mechanical shearing. gDNA samples must be fragmented prior to starting the Avida DNA workflow.

Fragmentation is only required for gDNA samples. If you are using cfDNA samples, proceed to [Chapter 4](#), “Library Preparation and Hybridization.”

NOTE

All liquid handling steps in this chapter are performed manually (no automation protocols).

Step 1. Prepare for shearing

NOTE

The shearing instructions provided here were optimized using a Covaris model E220 instrument and the 130- μ L Covaris microTUBE to generate fragment sizes of 150–300 bp. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve the same target gDNA fragment size.

- 1 Set up the Covaris instrument according to the manufacturer's instructions. Allow enough time (typically 30–60 minutes) for instrument degassing and water bath chilling before starting the protocol.
- 2 Dilute 10–100 ng of each gDNA sample with 1X low TE buffer to a final volume of 50 μ L. Vortex well to mix, then spin briefly. Keep the samples on ice.

Step 2. Shear the gDNA samples

- 1 For each gDNA sample, transfer the 50- μ L sample into a Covaris microTUBE.
- 2 Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.
- 3 Secure the microTUBE in the tube holder and shear the gDNA with the settings in [Table 15](#).

Table 15 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

Setting	High-quality gDNA	FFPE-derived gDNA
Duty Factor	10%	10%
Peak Incident Power (PIP)	175	175
Cycler per Burst	200	200
Treatment Time	2 \times 60 seconds (see two-round instructions below)	240 seconds
Bath Temperature	2° to 8°C	2° to 8°C

Use the steps below for two-round shearing of high-quality gDNA samples only:

- a Shear for 60 seconds
- b Spin the microTUBE for 10 seconds
- c Vortex the microTUBE at high speed for 5 seconds
- d Spin the microTUBE for 10 seconds
- e Repeat [step a](#) through [step d](#)
- 4 After shearing, put the Covaris microTUBE back into the loading and unloading station.
- 5 Spin the microTUBE briefly. While keeping the snap-cap on, insert a pipette tip through the pre-split septum, then slowly remove the sheared gDNA.
- 6 Transfer the sheared gDNA sample (approximately 50 μ L) to a strip tube sample well. Keep the samples on ice.

gDNA Fragmentation**Step 2. Shear the gDNA samples**

- 7 After transferring the gDNA sample, spin the microTUBE briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in [step 6](#) above.

The 50- μ L sheared gDNA samples are now ready for library preparation. Proceed directly to [Chapter 4](#), "Library Preparation and Hybridization."

This is not a stopping point.

4 Library Preparation and Hybridization

- Step 1. Prepare the NGS Workstation [30](#)
- Step 2. Prepare plates for the DNA samples, Hyb Buffer, and Library Wash Buffer [30](#)
- Step 3. Prepare the End Prep master mix [32](#)
- Step 4. Prepare the Ligation master mix [32](#)
- Step 5. Prepare the Adaptor mix [33](#)
- Step 6. Prepare the Hyb master mix [33](#)
- Step 7. Prepare the Library Prep master mix reagent source plate [34](#)
- Step 8. Load the NGS Workstation [36](#)
- Step 9. Run VWorks runset LibraryPrep_Avida_ILM [37](#)
- Step 10. Perform hybridization [39](#)

This chapter describes the steps for automated library preparation and hybridization plate setup with the Avida DNA panel. Libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

The Library Prep automation runset (LibraryPrep_Avida_ILM) directs the NGS Workstation to complete the steps for end-repair, dA-tailing, and ligation of adaptors. Then, the workstation prepares the plate of hybridization reactions for transfer to the thermal cyclor.

This chapter uses the reagents listed in [Table 16](#).

Table 16 Reagents for PCR-free library preparation and hybridization

Reagent	Usage Notes	Kit/Storage Location
End Prep Buffer (tube with purple cap)	Thaw at room temperature; vortex before use.	Avida DNA Reagent Box 1, stored at -20°C
End Prep Enzyme (tube with blue cap)	Place on ice or cold block just before use.	
Ligation Buffer (bottle)	Thaw at room temperature; vortex before use.	
Ligation Enzyme (tube with yellow cap)	Place on ice or cold block just before use.	
Adaptor for ILM (tube with orange cap)	Thaw on ice.	
Library Binding Beads (bottle)	Equilibrate to room temperature for at least 15 minutes; vortex before use. Allow bottle to sit upright for 5 minutes to let particles settle.	Avida Beads Box, stored at 4°C
Nuclease-free Water (bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature
Library Wash Buffer (bottle)	—	
Hyb Blocker (tube with red cap)	Thaw at room temperature.	Avida DNA Reagent Box 1, stored at -20°C
Hyb Buffer (bottle)	Heat to 37°C for at least 20 minutes before use. Once solution clears, keep at room temperature.	
Hyb Enhancer (amber tube with green cap)	Thaw at room temperature. If a precipitate is visible after thawing, thoroughly vortex until the solution clears.	
Avida DNA Panel	Thaw at room temperature then keep on ice.	Stored at -20°C

NOTE

Prior to starting a workflow, begin preheating the stock bottle of Hyb Wash Buffer 1 to ensure that it is sufficiently heated and solubilized prior to its use in [Chapter 5](#). The Hyb Wash Buffer 1 is provided in the Avida DNA and Duo Reagent Box 2.

- If a water bath is available, heat the stock bottle in a 50°C water bath for 10 minutes. Then, transfer the bottle to a heat block set to 50°C. Leave the bottle on the 50° heat block until use.
- If a water bath is not available, place the stock bottle on a heat block set to 50°C. Leave the bottle on the 50° heat block until use.

If the bottle does not fit in the heat block, place the bottle on top of the heat block, setting it on its side to increase surface area contact. A thermal cycler thermal block set to 50°C may be used in place of a heat block.

Step 1. Prepare the NGS Workstation

- 1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
- 2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
- 3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

Step 2. Prepare plates for the DNA samples, Hyb Buffer, and Library Wash Buffer

Automated runs may include 1, 2, 3, 4, 6, or 12 columns of DNA samples to be enriched for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Prepare the DNA sample plate

NOTE

On the VWorks form, if you plan to select one of the full-skirted plates for thermal cycling (Eppendorf twin.tec or Armadillo), make sure to use that same plate type for preparation of the DNA sample plate. The NGS Workstation uses the DNA sample plate as the thermal cycler plate.

If you select one of the semi-skirted plates for thermal cycling, then the NGS Workstation transfers the DNA samples to that plate at the end of the runset.

- 1 In the wells of a processing plate (Eppendorf twin.tec or Armadillo plate), add an appropriate quantity of DNA sample (based on sample type, see below) in a volume of 50 µL. If the sample volume is <50 µL, add Nuclease-free Water to bring the volume to 50 µL. See [Table 3](#) on page 11 for complete information on the DNA sample input requirements.
 - cfDNA samples: 1–100 ng
 - fragmented gDNA samples: 10–100 ng

Library Preparation and Hybridization

Step 2. Prepare plates for the DNA samples, Hyb Buffer, and Library Wash Buffer

NOTE

Load the DNA samples into the plate column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns.

- 2 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the plate on ice.

Prepare the Hyb Buffer plate

- 1 Heat the Hyb Buffer to 37°C in a water bath or heat block for at least 20 minutes, or until the Hyb Buffer is completely solubilized. If the Hyb Buffer bottle does not fit into the heat block, place the bottle on its side (to increase surface area contact) on top of the heat block.

If your laboratory does not have a water bath or heat block, you can heat the bottle of Hyb Buffer by placing it on top of the thermal block of a thermal cycler set to 37°C. More time may be required to completely solubilize the buffer with this technique.

- 2 Add the required volume of Hyb Buffer to the appropriate wells of a processing plate (Eppendorf twin.tec or Armadillo) based on the size of the Avida DNA panel.
 - 30 µL/well - For panels <500 kb (Focused Cancer Panel and Expanded Cancer Panel)
 - 31 µL/well - For panels ≥500 kb
- 3 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 4 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.
- 5 Keep the Hyb Buffer at room temperature until needed again in [“Step 2. Prepare the Capture Bead source plate”](#) on page 41.

Prepare the Library Wash Buffer plate

- 1 In the wells of an Agilent Deep Well plate, add 400 µL of Library Wash Buffer to each well of each column included in the run.
- 2 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Step 3. Prepare the End Prep master mix

- 1 Thaw the End Prep Buffer then vortex to mix. Inspect the buffer to make sure that no small particles are visible. If particles are visible, continue to vortex until they dissolve.
- 2 Prepare the appropriate volume of End Prep master mix at room temperature using volumes listed in [Table 17](#). Gently vortex the End Prep master mix at low speed, then briefly spin. Once prepared, keep the End Prep master mix on ice.

Table 17 Preparation of End Prep master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
End Prep Buffer (purple cap)	7 µL	98 µL	148 µL	204 µL	266 µL	406 µL	798 µL
End Prep Enzyme (blue cap)	3 µL	42 µL	64 µL	87 µL	114 µL	174 µL	342 µL
Total Volume	10 µL	140 µL	210 µL	291 µL	380 µL	580 µL	1140 µL

Step 4. Prepare the Ligation master mix

- 1 Thaw the Ligation Buffer at room temperature then vortex to mix. Inspect the buffer to make sure that no small particles are visible. If particles are visible, continue to vortex until they dissolve.
- 2 Prepare the appropriate volume of Ligation master mix at room temperature using volumes listed in [Table 18](#). Vortex the Ligation master mix, then briefly spin. Once prepared, keep the Ligation master mix on ice.

Table 18 Preparation of Ligation master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water (bottle)	16 µL	166 µL	314 µL	456 µL	602 µL	920 µL	1800 µL
Ligation Buffer (bottle)	25 µL	260 µL	490 µL	712 µL	942 µL	1436 µL	2814 µL
Ligation Enzyme (yellow cap)	6 µL	62 µL	118 µL	172 µL	226 µL	344 µL	679 µL
Total Volume	47 µL	490 µL	920 µL	1340 µL	1770 µL	2700 µL	5290 µL

Step 5. Prepare the Adaptor mix

- 1 Thaw the Adaptor for ILM on ice then vortex to mix.
- 2 Prepare the appropriate volume of Adaptor mix on ice using the volumes listed in [Table 19](#). Vortex the Adaptor mix, then briefly spin. Keep the Adaptor mix on ice.

Table 19 Preparation of Adaptor mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water (bottle)	2.5 µL	36 µL	54 µL	74 µL	96 µL	146 µL	286 µL
Adaptor for ILM (orange cap)	5 µL	73 µL	107 µL	147 µL	193 µL	293 µL	573 µL
Total Volume	7.5 µL	110 µL	160 µL	220 µL	290 µL	440 µL	860 µL

Step 6. Prepare the Hyb master mix

- 1 Thaw the Hyb Blocker, Hyb Enhancer, and Avida Panel at room temperature. Transfer the Avida Panel to ice once thawed.
- 2 Prepare the appropriate volume of Hyb master mix at room temperature using volumes listed in [Table 20](#) or [Table 21](#) (depending on panel size). Vortex the Hyb master mix, then briefly spin. Keep the Hyb master mix at room temperature.
 - [Table 20](#) - For panels <500 kb (Focused Cancer Panel and Expanded Cancer Panel)
 - [Table 21](#) - For panels ≥500 kb

Table 20 Preparation of Hyb master mix for panels <500 kb

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water (bottle)	17.5 µL	222 µL	390 µL	554 µL	718 µL	1050 µL	2036 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	102 µL	150 µL	290 µL
Hyb Enhancer (amber tube with green cap)	6 µL	76 µL	134 µL	190 µL	246 µL	360 µL	698 µL
Avida DNA panel	4 µL	50 µL	90 µL	126 µL	164 µL	240 µL	466 µL
Total Volume	30 µL	380 µL	670 µL	950 µL	1230 µL	1800 µL	3490 µL

Table 21 Preparation of Hyb master mix for panels ≥ 500 kb

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water (bottle)	17.5 μ L	224 μ L	392 μ L	556 μ L	724 μ L	1056 μ L	2046 μ L
Hyb Blocker (red cap)	2.5 μ L	32 μ L	56 μ L	80 μ L	104 μ L	150 μ L	292 μ L
Hyb Enhancer (amber tube with green cap)	5 μ L	64 μ L	112 μ L	158 μ L	206 μ L	302 μ L	584 μ L
Avida DNA panel	4 μ L	52 μ L	90 μ L	126 μ L	166 μ L	242 μ L	468 μ L
Total Volume	29 μL	370 μL	650 μL	920 μL	1200 μL	1750 μL	3390 μL

Step 7. Prepare the Library Prep master mix reagent source plate

The Library Prep master mix reagent source plate is an Agilent Deep Well plate containing the Avida reagents used in the Library Prep runset.

- Using an Agilent Deep Well plate, prepare the master mix reagent source plate for the Library Prep runset by adding the required reagents to the appropriate wells. Keep the plate on ice during and after preparation.

Table 22 lists the required volumes and well position(s) for each reagent based on the number of columns in the run. Figure 3 shows the configuration of the reagent source plate.

Table 22 Volumes (per well) for the Library Prep master mix reagent source plate based on number of columns in the run

Master Mix Solution	Position on Source Plate	Volume of master mix added per well of source plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Library Binding Beads	Column 1 (A1-H1)	100 μ L	190 μ L	280 μ L	370 μ L	550 μ L	1090 μ L
End Prep master mix	Column 2 (A2-H2)	17 μ L	27 μ L	36 μ L	47 μ L	71 μ L	139 μ L
Adaptor mix	Column 3 (A3-H3)	13 μ L	20 μ L	27 μ L	36 μ L	54 μ L	106 μ L
Ligation master mix	Column 4 (A4-H4)	58 μ L	109 μ L	160 μ L	211 μ L	318 μ L	624 μ L
Hyb master mix	Column 5 (A5-H5)	46 μ L	80 μ L	114 μ L	148 μ L	216 μ L	420 μ L

Library Preparation and Hybridization

Step 7. Prepare the Library Prep master mix reagent source plate

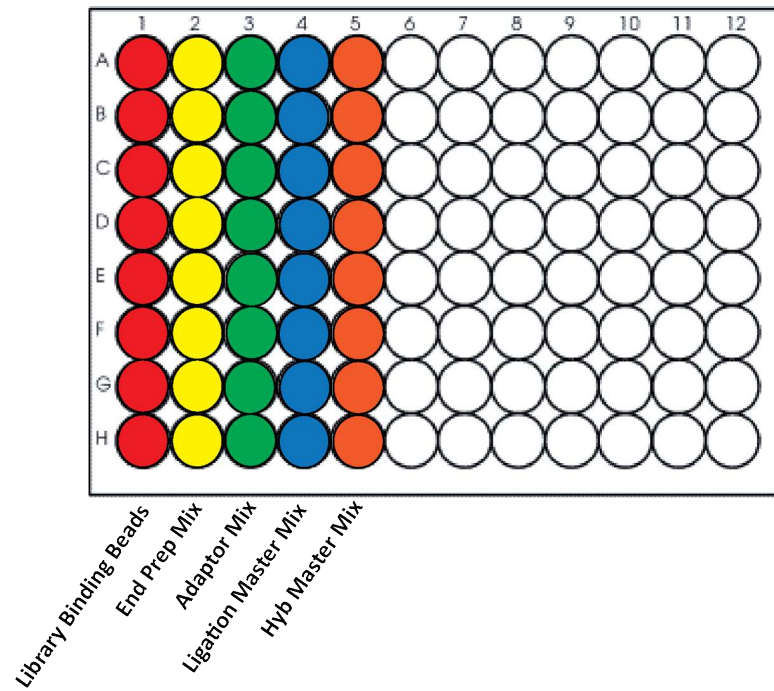


Figure 3 Configuration of the Library Prep master mix source plate for runset LibraryPrep_Avida_ILM

- 2 Seal the reagent source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate at 50 × g for 1 second to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the plate on ice.

NOTE

The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Step 8. Load the NGS Workstation

- Using the plate orientations shown in [Figure 4](#), load the Labware MiniHub according to [Table 23](#).

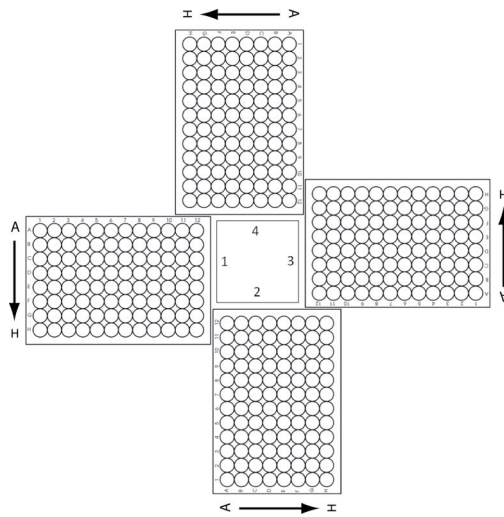


Figure 4 Agilent Labware MiniHub plate orientation.

Table 23 Initial MiniHub configuration for LibraryPrep_Avida_ILM runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing	–	–	–
Shelf 4	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing	–	–	–
Shelf 3	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing	–	–	–
Shelf 2	New tip box	Hyb Buffer plate prepared on page 31	–	–
Shelf 1 (Bottom)	Empty tip box	Library Wash Buffer plate prepared on page 31	–	Empty tip box

- Load the Bravo deck according to [Table 24](#).

Library Preparation and Hybridization

Step 9. Run VWorks runset LibraryPrep_Avida_ILM

Table 24 Initial Bravo deck configuration for LibraryPrep_Avida_ILM runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
5	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing
7	DNA sample plate prepared on page 30
9	Library prep master mix source plate prepared on page 34

3 Load the BenchCel Microplate Handling Workstation according to [Table 25](#).

Table 25 Initial BenchCel configuration for LibraryPrep_Avida_ILM runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	–	–	–
2	2 Tip boxes	–	–	–
3	2 Tip boxes	–	–	–
4	3 Tip boxes	–	–	–
6	4 Tip boxes	–	–	–
12	7 Tip boxes	–	–	–

Step 9. Run VWorks runset LibraryPrep_Avida_ILM

- 1 On the Avida DNA VWorks form, under **Select protocol to execute**, select the **LibraryPrep_Avida_ILM** runset.
- 2 Under **Select labware for thermal cycling**, select the specific type of PCR plate to be used for the hybridization reactions.

NOTE

If you select one of the semi-skirted plates for thermal cycling, VWorks will prompt you at the end of the runset when it is time to add the empty semi-skirted plate to Bravo deck position 4.

If you select one of the full-skirted plates (Eppendorf twin.tec or Armadillo) make sure that the sample plate containing the input DNA samples was prepared using that same plate type (see [“Prepare the DNA sample plate”](#) on page 30).

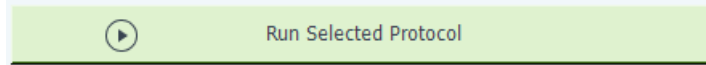
- 3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
- 4 Click **Display Initial Setup**.

Display Initial Setup

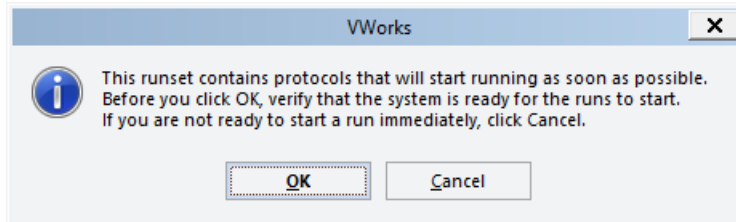
Library Preparation and Hybridization

Step 9. Run VWorks runset LibraryPrep_Avida_ILM

- 5 Verify that the NGS Workstation has been set up as displayed on the right side of the form.
- 6 When verification is complete, click **Run Selected Protocol**.

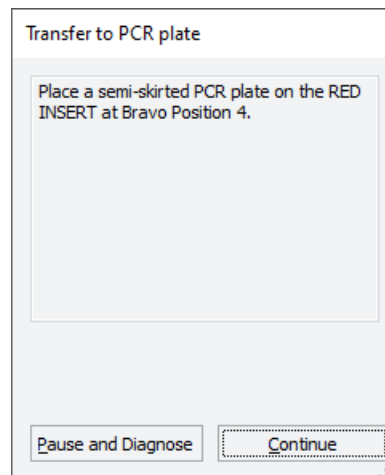


- 7 When ready to begin the run, click **OK** in the following window.

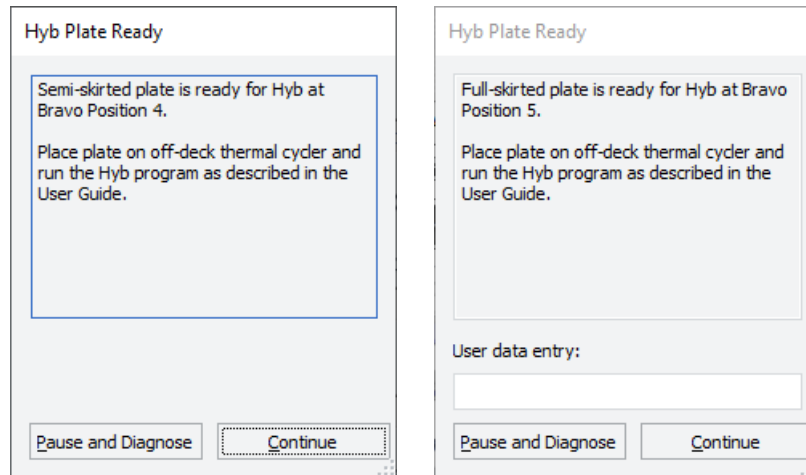


Running the LibraryPrep_Avida_ILM runset takes approximately 3 hours. During the runset, the NGS Workstation completes the PCR-free library preparation, including end-repair, dA-tailing, and ligation of the adaptor.

- 8 While the runset is running, pre-program the thermal cycler for the hybridization thermal cycler program as directed in [Table 26](#) in “[Step 10. Perform hybridization](#)” on page 39.
- 9 If you selected a semi-skirted thermal cycling plate in [step 2](#), then VWorks prompts you near the end of the runset to add an empty PCR plate to position 4 of the Bravo deck. The prompt is shown below. After adding the plate, click **Continue**. The workstation then prepares the plate of hybridization reactions for transfer to the thermal cycler.



- 10 When prompted by VWorks that the Hyb plate is ready (the two possible prompts are shown below) at the completion of the LibraryPrep_Avida_ILM runset, remove the PCR plate from the Bravo deck as instructed in the prompt. This plate contains the hybridization reactions. After removing the plate, click **Continue**.



- 11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Proceed to [“Step 10. Perform hybridization.”](#)

Step 10. Perform hybridization

- 1 While the LibraryPrep_Avida_ILM runset is in progress, pre-program the thermal cycler (with the heated lid ON) with the program in [Table 26](#). Set the heated lid to 103°C. Start the program. Once the lid is heated, pause the program.

Table 26 Thermal cycler program for hybridization

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	≥2.5°C/second ramp down to 60°C	
Step 3	60°C	60 minutes
Step 4	60°C	Hold

NOTE

The thermal cycler program in [Table 26](#) uses a 60-minute hybridization which is sufficient for most applications. For panels >500 kb, a longer hybridization time (up to 16 hours) may improve capture efficiency.

- 2 Transfer the sealed plate containing the hybridization reactions to the pre-programmed thermal cycler. Resume the pre-programmed thermal cycler program for hybridization ([Table 26](#)).
- 3 When the hybridization thermal cycler program has approximately 30 minutes remaining, start preparing for the Capture & Wash runset by proceeding to [Chapter 5](#), “Capture and Washing” starting on [page 40](#).

5 Capture and Washing

- Step 1. Prepare the NGS Workstation [41](#)
- Step 2. Prepare the Capture Bead source plate [41](#)
- Step 3. Prepare remaining plates for target capture and washing [44](#)
- Step 4. Load the NGS Workstation [45](#)
- Step 5. Run the target capture and washing runset or protocol [46](#)

This chapter describes the steps for automated target capture and washing using Capture Beads coated with Streptavidin.

The Avida DNA VWorks form offers two options for automated target capture and washing: the TargetCapture_Avida runset and the Capture&Wash_Avida protocol. Both of these automation programs direct the NGS Workstation to complete the steps for capturing the library DNA hybrids with Capture Beads, washing the bead-bound DNA, and eluting the bead-bound DNA. The TargetCapture_Avida runset, however, completes the additional steps needed for preparation of the Capture Beads (upstream of target capture), while the Capture&Wash_Avida protocol is intended for use with Capture Beads that are prepared manually.

NOTE

Initiate the instructions in this chapter when the hybridization thermal cycler program has approximately 30 minutes remaining. After starting the Capture and Wash protocol or runset ([page 46](#)), the VWorks software prompts you when it is time to add the completed hybridization reaction plate to the Bravo deck.

This chapter uses the reagents listed in [Table 27](#).

Table 27 Reagents for capture and washing

Reagent	Usage Notes	Kit/Storage Location
Hyb Buffer (bottle)	Following use previous chapter, keep at room temperature. Vortex before use.	Avida DNA Reagent Box 1, stored at -20°C
Capture Beads (tube with amber cap)	Thoroughly vortex to mix before use.	Avida Beads Box, stored at 4°C
Nuclease-free Water (bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature
Hyb Wash Buffer 1 (bottle)	Heat the stock bottle at 50°C in water bath or heat block until use.	
Hyb Wash Buffer 2 (bottle)	—	
Resuspension Buffer (bottle)	—	

Step 1. Prepare the NGS Workstation

- 1 On the Labware MiniHub, leave in place the tip boxes located on shelves 1 and 2 of cassette 1 that were used during the previous runset (LibraryPrep_Avida_ILM). Clear the Labware MiniHub and BenchCel of all remaining plates and tip boxes.
- 2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
- 3 Place the silver Deep Well plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to the Deep Well source plate wells during the Capture & Wash runset. When loading a source plate on the silver insert, make sure the plate is seated properly to ensure proper heat transfer.
- 4 Pre-set the temperature of Bravo deck position 4 to 60°C using the Inheco Multi TEC control touchscreen, as described in [Setting the Temperature of Bravo Deck Heat Blocks](#). Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
- 5 Pre-set the temperature of Bravo deck position 6 to 55°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
- 6 Retract the tip box stripper pins on the disposable-tip pipette head.

Retracting the stripper pins enables single and partial head mode pipette tip loading. You can release the tip box stripper pins at the end of the runset. Refer to the Bravo Platform User Guide, publication SD-V1000376, for detailed instructions.

Step 2. Prepare the Capture Bead source plate

The Capture Beads need to be washed before they can be used for target capture and washing. The Avida DNA VWorks form supports two approaches for preparing washed Capture Beads: automated preparation and manual preparation.

Follow the instructions in [Option 1: Prepare the Capture Bead source plate for automated bead prep](#) to use automation to prepare the beads.

Follow the instructions in [Option 2: Prepare the Capture Bead source plate using pre-washed beads](#) to prepare the beads manually.

Option 1: Prepare the Capture Bead source plate for automated bead prep

The Capture Bead source plate is a processing plate containing the reagents needed to wash the Capture Beads prior to their addition to the DNA library hybrids. This option is for use with the TargetCapture_Avida runset.

- 1 Thaw or prepare the reagents as described in [Table 27](#) on page 40.

CAUTION

Hyb Wash Buffer 1 may become cloudy or form crystals during storage. Make sure that the solution clears during the 50°C heating. Thoroughly vortex or invert the stock bottle before use.

- 2 Using a processing plate (Eppendorf twin.tec or Armadillo), prepare the Capture Bead source plate for the TargetCapture_Avida runset by adding the required reagents to the appropriate wells. Keep the plate at room temperature during and after preparation.

Capture and Washing

Step 2. Prepare the Capture Bead source plate

Table 28 lists the required volumes and well position(s) for each reagent based on the number of columns in the run. Figure 5 shows the configuration of the Capture Bead source plate.

Table 28 Volumes (per well) for the Capture Bead source plate based on number of columns in the run

Reagent	Position on Source Plate	Volume of master mix added per well of source plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Capture Beads	Column 1 (A1-H1)	16 µL	24 µL	28 µL	36 µL	56 µL	112 µL
Hyb Wash Buffer 1 preheated to 50°C	Column 2 (A2-H2)	160 µL	160 µL	160 µL	160 µL	160 µL	160 µL
Hyb Wash Buffer 1 preheated to 50°C	Column 3 (A3-H3)	160 µL	160 µL	160 µL	160 µL	160 µL	160 µL
Hyb Buffer	Column 4 (A4-H4)	30 µL	40 µL	40 µL	50 µL	70 µL	130 µL

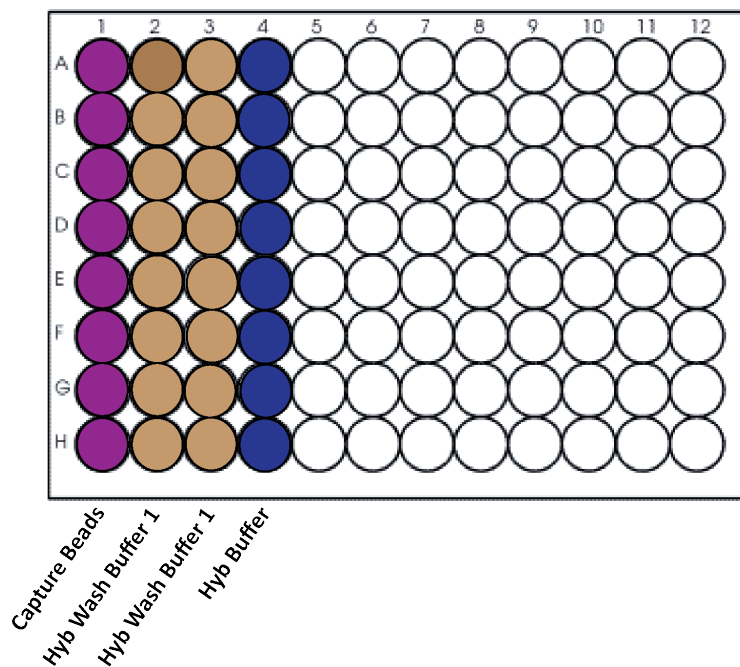


Figure 5 Configuration of the Capture Bead source plate for runset TargetCapture_Avida

- Seal the Capture Bead source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- Centrifuge the plate at 50 × g for 1 second to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Proceed to [“Step 3. Prepare remaining plates for target capture and washing”](#) on page 44.

NOTE

The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Capture and Washing

Step 2. Prepare the Capture Bead source plate

Option 2: Prepare the Capture Bead source plate using pre-washed beads

The Capture Bead source plate is a processing plate containing pre-washed Capture Beads that were manually prepared. This option is for use with the Capture&Wash_Avida protocol.

- 1 Thaw or prepare the reagents as described in [Table 27](#) on page 40.

CAUTION

Hyb Wash Buffer 1 may become cloudy or form crystals during storage. Make sure that the solution clears during the 50°C heating. Thoroughly vortex or invert the stock bottle before use.

- 2 Thoroughly vortex the Hyb Buffer that has been kept at room temperature.
- 3 Thoroughly vortex the Capture Beads stock to make sure the solution is homogeneous.
- 4 Refer to [Table 29](#) to determine the required volume of Capture Beads based on the number of columns in the run. Add that volume of Capture Beads to a 1.5-mL tube.
- 5 Place the tube of Capture Beads on the magnet for 1 minute or until the solution becomes clear. Remove and discard the supernatant. Remove the tube from the magnet.
- 6 Refer to [Table 29](#) to determine the appropriate volume of preheated Hyb Wash Buffer 1 to use for washing the Capture Beads. Add that volume of Hyb Wash Buffer 1 to the tube of Capture Beads. Mix well by vortexing or by pipetting up and down at least 15–20 times.

Make sure the beads are fully resuspended and well mixed. When working with wash volumes >100 µL, mixing by pipetting is more effective than vortexing.

- 7 Briefly spin the tube of Capture Beads, then place the tube on the magnet for 1 minute or until the solution becomes clear. Remove and discard the supernatant.
- 8 Perform an additional wash by repeating [step 6](#) through [step 7](#) for a total of two washes.

Place the Hyb Wash Buffer 1 back on the 50°C heat block or water bath.

- 9 Remove the tube of Capture Beads from the magnet. Resuspend the beads in the appropriate volume of Hyb Buffer listed in [Table 29](#). Mix by pipetting up and down 15–20 times (avoid creating excess bubbles).

Table 29 Volumes for preparing washed Capture Beads in a 1.5-mL tube

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Capture Beads	8 µL	128 µL	192 µL	224 µL	288 µL	448 µL	896 µL
Hyb Wash Buffer 1 preheated to 50°C	100 µL	100 µL	100 µL	200 µL	200 µL	300 µL	600 µL
Hyb Buffer	8 µL	128 µL	192 µL	224 µL	288 µL	448 µL	896 µL

- 10 In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 8 µL of washed Capture Beads to each well of each column included in the run.
- 11 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 12 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Step 3. Prepare remaining plates for target capture and washing

The remaining plates needed for the TargetCapture_Avida runset or Capture&Wash_Avida protocol contain reagents for washing and resuspending the DNA libraries.

Prepare the Hyb Wash Buffer 1 plate

- 1 In the wells of an Agilent Deep Well plate, add 300 μ L of preheated Hyb Wash Buffer 1 to each well of each column included in the run.
- 2 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Prepare the Hyb Wash Buffer 2 plate

- 1 In the wells of an Agilent Deep Well plate, add 160 μ L of Hyb Wash Buffer 2 to each well of each column included in the run.
- 2 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Prepare the Resuspension Buffer plate

- 1 In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 24 μ L of Resuspension Buffer to each well of each column included in the run.
- 2 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

At the end of the Capture&Wash_Avida runset, you can keep this plate for later use as the 1X Low TE Buffer plate in the AMPureXP_Cleanup protocol. See [“Step 2. Prepare the 1X Low TE Buffer plate”](#) on page 56.

Step 4. Load the NGS Workstation

- 1 Load the Labware MiniHub according to the appropriate table below. Use the plate orientations shown in [Figure 4](#) on page 36.
 - If you prepared the Capture Bead source plate using [Option 1: Prepare the Capture Bead source plate for automated bead prep](#), see [Table 30](#).
 - If you prepared the Capture Bead source plate using [Option 2: Prepare the Capture Bead source plate using pre-washed beads](#), see [Table 31](#).

Table 30 Initial MiniHub configuration for TargetCapture_Avida runset (for automated bead prep)

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty processing plate (Eppendorf twin.tec or Armadillo)	—	—	—
Shelf 4	Resuspension Buffer plate prepared on page 44	—	—	—
Shelf 3	Empty processing plate (Eppendorf twin.tec or Armadillo)	—	—	—
Shelf 2	Tip box from Library Prep runset (new tips in column 12)	—	Hyb Wash Buffer 2 plate prepared on page 44	—
Shelf 1 (Bottom)	Tip box from Library Prep runset (empty in column 12)	—	—	Empty tip box

Table 31 Initial MiniHub configuration for Capture&Wash_Avida protocol (for manual bead prep)

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	Resuspension Buffer plate prepared on page 44	—	—	—
Shelf 3	Empty processing plate (Eppendorf twin.tec or Armadillo)	—	—	—
Shelf 2	Tip box from Library Prep runset (new tips in column 12)	—	Hyb Wash Buffer 2 plate prepared on page 44	—
Shelf 1 (Bottom)	Tip box from Library Prep runset (empty in column 12)	—	—	Empty tip box

Capture and Washing

Step 5. Run the target capture and washing runset or protocol

- 2 Load the Bravo deck according to [Table 32](#).

Table 32 Initial Bravo deck configuration for TargetCapture_Avida runset or Capture&Wash_Avida protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
5	Capture Bead source plate prepared on page 41 (using option 1) or page 43 (using option 2)
6	Hyb Wash Buffer 1 plate prepared on page 44 , seated in silver insert
7	Empty at start of runset (add hybridization plate from the thermal cycler when prompted by VWorks)

- 3 Load the BenchCel Microplate Handling Workstation according to [Table 33](#).

Table 33 Initial BenchCel configuration for Capture and Wash

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	3 Tip boxes	—	—	—
6	4 Tip boxes	—	—	—
12	7 Tip boxes	—	—	—

Step 5. Run the target capture and washing runset or protocol

NOTE

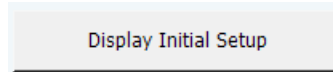
Approximately 20 minutes after starting the selected runset or protocol for target capture and washing, VWorks opens a prompt to add the hybridization plate to the Bravo deck. Shortly after, VWorks opens another prompt to remove the hybridization plate. Make sure an operator is present to perform these actions. The remainder of the runset/protocol takes approximately 40 minutes to complete.

- 1 On the Avida DNA VWorks form, under **Select protocol to execute**, select the appropriate option from the menu.
 - If you prepared the Capture Bead source plate using [Option 1: Prepare the Capture Bead source plate for automated bead prep](#), select runset **TargetCapture_Avida**.
 - If you prepared the Capture Bead source plate using [Option 2: Prepare the Capture Bead source plate using pre-washed beads](#), select protocol **Capture&Wash_Avida**.
- 2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

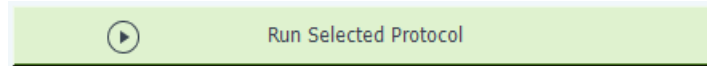
Capture and Washing

Step 5. Run the target capture and washing runset or protocol

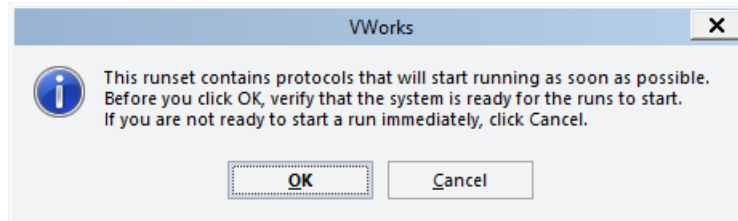
- 3 Click **Display Initial Setup**.



- 4 Verify that the NGS Workstation has been set up as displayed on the right side of the form.
- 5 When verification is complete, click **Run Selected Protocol**.



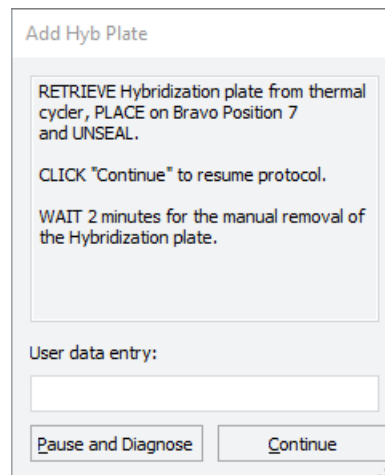
- 6 If the following prompt opens, click **OK** to continue.



- 7 When prompted by VWorks as shown below, transfer the hybridization plate from the thermal cyclers to position 7 of the Bravo deck. After adding the plate, click **Continue**.

NOTE

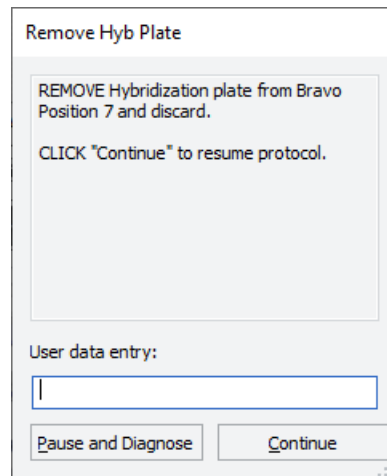
If the hybridization thermal cycler program has not yet reached the 60°C hold step, wait until that step is reached before transferring the plate from the thermal cycler to the Bravo deck.



Capture and Washing

Step 5. Run the target capture and washing runset or protocol

- When prompted by VWorks for a second time, as shown below, remove and discard the now empty hybridization plate from position 7 of the Bravo deck. After removing the plate, click **Continue**.



- When the runset or protocol is complete, the plate containing the resuspended Capture Beads with captured DNA is located in the red insert at position 4 of the Bravo deck. Transfer the plate to ice.

To avoid bead settling, proceed immediately to [Chapter 6](#), "Indexing PCR" starting on [page 49](#). The plate of resuspended Capture Beads with captured DNA will be loaded on the Bravo deck (see [Table 37](#) on page 52) for the PCR_Avida_ILM protocol.

6 Indexing PCR

- Step 1. Prepare the NGS Workstation for the PCR_Avida_ILM protocol [50](#)
- Step 2. Prepare the 2X Amplification Master Mix source plate [50](#)
- Step 3. Prepare the Index Primer Pairs plate [51](#)
- Step 4. Load the NGS Workstation for the PCR_Avida_ILM protocol [52](#)
- Step 5. Run VWorks protocol PCR_Avida_ILM [53](#)
- Step 6. Perform PCR cycling [53](#)

This chapter describes the steps for automated plate setup for the indexing PCR reactions.

The PCR automation protocol (PCR_Avida_ILM) directs the NGS Workstation to complete the steps for preparing the PCR plate. After the PCR plate is prepared by the workstation, you transfer the plate to a thermal cycler for amplification. The PCR reactions use index primer pairs to generate amplified, indexed libraries.

This chapter uses the reagents listed in [Table 34](#).

Table 34 Reagents for indexing PCR

Reagent	Usage Notes	Kit/Storage Location
2X Amplification Mastermix (bottle)	Thaw on ice.	Avida DNA Reagent Box 1, stored at -20°C
Avida Index Primer Pairs	Primer pairs are provided in a 96-well plate. Thaw plate on ice.	Stored at -20°C
Nuclease-free Water (bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature

CAUTION

Take precautions to avoid amplicon contamination during setup of the indexing PCR reactions. Review [“Important practices for preventing contamination”](#) on page 8.

Step 1. Prepare the NGS Workstation for the PCR_Avida_ILM protocol

- 1 Clear the Labware MiniHub and BenchCel of plates and tip boxes.
- 2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

Step 2. Prepare the 2X Amplification Master Mix source plate

- 1 Using a processing plate (Eppendorf twin.tec or Armadillo), prepare the 2X Amplification Master Mix source plate for the PCR_Avida_ILM protocol by adding the required volume of 2X Amplification Master Mix to the appropriate wells, as indicated in [Table 35](#). Keep the plate on ice during and after preparation.

For runs with 1, 2, 3, 4, 6 or 12 columns, all wells in column 1 need to be filled with the volume of 2X Amplification Mastermix indicated in [Table 35](#). For runs with 12 columns, all wells of column 2 also need to be filled with the indicated volume. The final configuration of the source plate is shown in [Figure 6](#).

At the end of the PCR_Avida_ILM protocol, you can retain the source plate for use in the TS_D1000 protocol. See [“Prepare the workstation and Sample Buffer source plate”](#) on page 59.

Table 35 Preparation of the 2X Amplification Mastermix source plate for PCR_Avida_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per well of source slate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
2X Amplification Master Mix	Column 1 (A1-H1)	29.0 µL	54.0 µL	79.0 µL	104.0 µL	154.0 µL	154.0 µL
	Column 2 (A2-H2)	—	—	—	—	—	154.0 µL

Indexing PCR

Step 3. Prepare the Index Primer Pairs plate

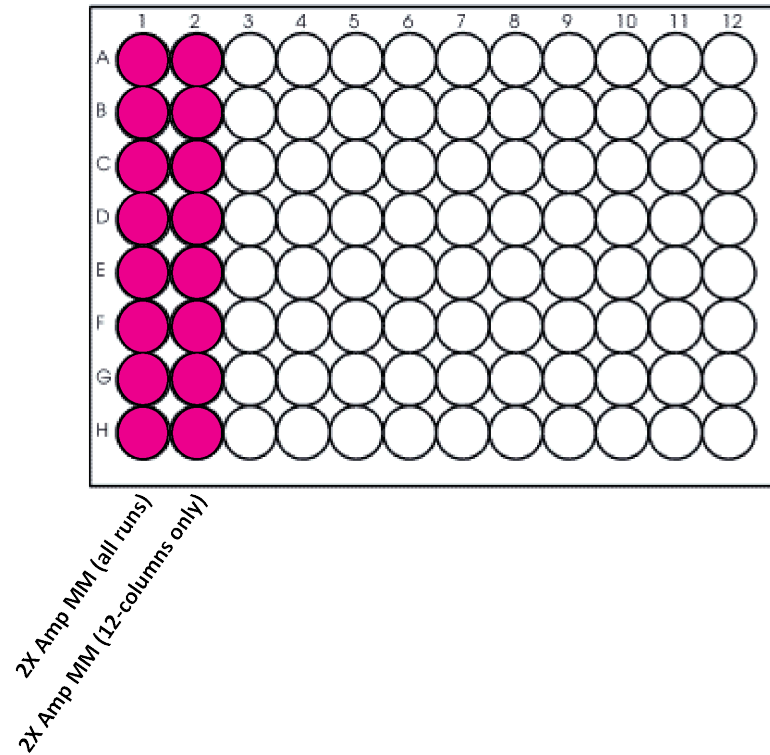


Figure 6 Configuration of the 2X Amplification Master Mix source plate (Eppendorf twin.tec or Armadillo plate) for protocol PCR_Avida_ILM. For all runs, the 2X Amplification Mastermix is distributed to all wells of column 1. For runs with 12 columns, the 2X Amplification Mastermix is also distributed to all wells of column 2.

- 2 Seal the master mix source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Step 3. Prepare the Index Primer Pairs plate

- 1 Using a multichannel pipette, transfer 5 μ L of each Avida Index Primer Pair from the 96-well plate in which the primer pairs are provided into the PCR plate to be used for the indexing PCR thermal cycling. Make sure to maintain the same well location for each primer pair when transferring to the PCR plate. Keep the PCR plate on ice.

Step 4. Load the NGS Workstation for the PCR_Avida_ILM protocol

- 1 Load the Labware MiniHub according to [Table 36](#), using the plate orientations shown in [Figure 4](#) on page 36.

Table 36 Initial MiniHub configuration for PCR_Avida_ILM protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

- 2 Load the Bravo deck according to [Table 37](#).

Table 37 Initial Bravo deck configuration for PCR_Avida_ILM protocol

Location	Content
4	Resuspended Capture Beads with captured DNA in processing plate (unsealed) seated in red insert
5	2X Amplification Master Mix source plate prepared on page 50
6	Index Primer Pairs plate prepared on page 51 (seated in red insert if plate is semi-skirted)

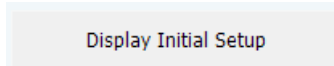
- 3 Load the BenchCel Microplate Handling Workstation according to [Table 38](#).

Table 38 Initial BenchCel configuration for PCR_Avida_ILM protocol

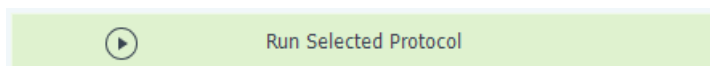
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Step 5. Run VWorks protocol PCR_Avida_ILM

- 1 On the Avida DNA VWorks form, under **Select protocol to execute**, select the **PCR_Avida_ILM** protocol.
- 2 Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.
- 3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
- 4 Click **Display Initial Setup**.



- 5 Verify that the NGS Workstation has been set up as displayed on the right side of the form.
- 6 When verification is complete, click **Run Selected Protocol**.



Running the protocol takes approximately 15 minutes. Once complete, the PCR reactions – containing captured DNA, 2X Amplification Master Mix, and Avida Index Primer Pairs – are located in the PCR plate at position 6 of the Bravo deck.

- 7 Proceed to **“Step 6. Perform PCR cycling”** below to start preparing the thermal cycler for PCR cycling.

Step 6. Perform PCR cycling

- 1 While the PCR_Avida_ILM runset is in progress, pre-program the thermal cycler to run the program shown in [Table 39](#). Set the heated lid to 103°C. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 39 Thermal cycler program for indexing PCR

Step	Number of Cycles	Temperature	Time
Initial denaturation	1	98°C	45 seconds
Amplification stage 1	5	98°C	10 seconds
		63°C	30 seconds
		72°C	30 seconds
Amplification stage 2	Variable, see Table 40 for recommendations	98°C	10 seconds
		72°C	1 minute
Final extension	1	72°C	1 minute
Final hold	1	4°C	Hold

Indexing PCR

Step 6. Perform PCR cycling

Table 40 Indexing PCR cycle number recommendations for Amplification stage 2

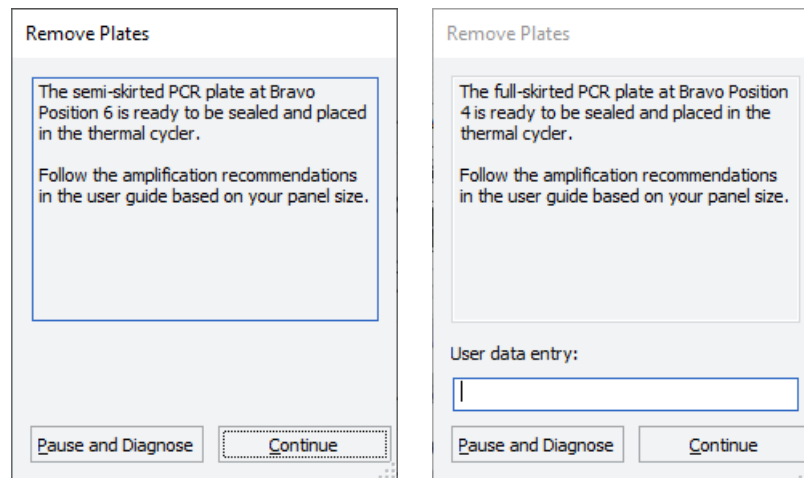
Panel size	Number of cycles	Notes
>100 kb	16 or fewer	For the Avida DNA Expanded Cancer Panel (345 kb): 14 cycles
50–100 kb	17	—
10–50 kb	18	Includes the Avida DNA Focused Panel (26 kb)
<10 kb	19	—

NOTE

The number of cycles recommended in [Table 40](#) are based on a sample input of 10 ng of cfDNA. If using other sample types or input quantities, you may need to optimize the number of cycles using the points below for guidance.

- High-quality gDNA samples typically require 1–2 cycles more than cfDNA samples of the same input quantity.
- Low-quality gDNA samples (e.g., FFPE-derived gDNA) may require more cycles than high-quality gDNA samples. The number of additional cycles depends on the degree of sample degradation, with greater degradation necessitating more cycles.
- A sample input >50 ng typically requires 1–2 fewer cycles than a sample input of 10 ng.

- 2 When prompted by VWorks that the PCR plate is ready (the two possible prompts are shown below), remove the PCR plate from the Bravo deck as instructed in the prompt. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.



- 3 Place the plate in the thermal cycler. Resume the thermal cycler program in [Table 39](#) on page 53.
- 4 While the indexing PCR thermal cycler program is running, start preparing for Library Purification by proceeding to [Chapter 7](#), "Library Purification, Quality Assessment, and Sample Pooling" starting on [page 40](#).

Stopping Point If you do not continue to the next step, store the indexed libraries at 4°C overnight by leaving the plate in the thermal cycler at the 4°C hold step.

7 Library Purification, Quality Assessment, and Sample Pooling

Library Purification	56
Step 1. Prepare the AMPure XP bead plate	56
Step 2. Prepare the 1X Low TE Buffer plate	56
Step 3. Prepare the NGS Workstation	56
Step 4. Run VWorks protocol AMPureXP_Cleanup	58
Library Quantity and Quality Assessment	59
Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape	59
Option 2: Analysis using an equivalent platform (non-automated)	63
Sample Pooling for Multiplexed Sequencing (optional)	65
Option 1: Pool samples at equal volumes (non-automated)	65
Option 2: Pool samples at varying volumes (automated or non-automated)	65

This chapter describes the steps for automated library purification, quality analysis plate setup, and optional sample pooling.

The Library Cleanup automation protocol (AMPureXP_Cleanup) is used to purify the libraries. The TapeStation DNA 1000 automation protocol (TS_D1000) directs the workstation to set up the analysis plate for the DNA 1000 assay on the TapeStation. The automation protocol for sample pooling (Aliquot_Captures) can be used to pool samples prior to sequencing.

This chapter uses the reagents listed in [Table 41](#).

Table 41 Reagents for library purification and quality assessment

Reagent	Usage Notes	Kit/Storage Location
AMPure XP Beads	Equilibrate at room temperature for 30 minutes.	Not provided
100% ethanol	—	Not provided
1X Low TE Buffer	—	Not provided
Qubit HS dsDNA Assay Kit	—	Not provided
Nucleic acid analysis kit	—	Not provided

Library Purification

In this step, the NGS Workstation transfers AMPure XP beads to the indexed DNA sample plate and then collects and washes the bead-bound DNA.

Step 1. Prepare the AMPure XP bead plate

- 1 Verify that the AMPure XP bead suspension is at room temperature.
- 2 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. Do not freeze.
- 3 In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 45 μ L of bead suspension each well of each column included in the run.

You can use a reagent reservoir and multichannel pipette to perform this step.

Step 2. Prepare the 1X Low TE Buffer plate

- 1 In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 30 μ L of 1X Low TE Buffer to each well of each column included in the run.

You can use the same processing plate that was used earlier as the Resuspension Buffer plate in the Capture&Wash_Avida runset. See [“Prepare the Resuspension Buffer plate”](#) on page 44.

- 2 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the plate on ice.

Step 3. Prepare the NGS Workstation

- 1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
- 2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
- 3 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
- 4 Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 80% ethanol.
- 5 Load the Labware MiniHub according to [Table 42](#), using the plate orientations shown in [Figure 4](#) on page 36.

Library Purification, Quality Assessment, and Sample Pooling

Step 3. Prepare the NGS Workstation

Table 42 Initial MiniHub configuration for AMPureXP_Cleanup protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	AMPure XP bead plate prepared on page 56	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty processing plate	—	—
Shelf 2	—	1X Low TE Buffer plate prepared on page 56	—	—
Shelf 1 (Bottom)	—	80% ethanol reservoir prepared on page 56	—	Empty tip box

6 Load the Bravo deck according to [Table 43](#).

Table 43 Initial Bravo deck configuration for AMPureXP_Cleanup protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
9	Amplified DNA libraries in unsealed PCR plate; seated in a red insert if using a semi-skirted plate (PCR plate type must be specified on setup form under step 2)

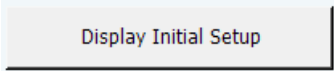
7 Load the BenchCel Microplate Handling Workstation according to [Table 44](#).

Table 44 Initial BenchCel configuration for AMPureXP_Cleanup protocol

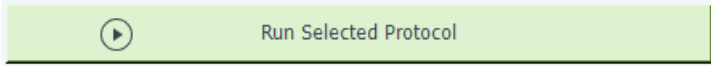
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

Step 4. Run VWorks protocol AMPureXP_Cleanup

- 1 On the Avida DNA VWorks form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Post-Capture PCR)** protocol.
- 2 Under **Select labware for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.
- 3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
- 4 Click **Display Initial Setup**.

A rectangular button with a light gray background and a thin black border. The text "Display Initial Setup" is centered in a dark gray font.

- 5 Verify that the NGS workstation has been set up as displayed on the right side of the form.
- 6 When verification is complete, click **Run Selected Protocol**.

A rectangular button with a light green background and a thin black border. The text "Run Selected Protocol" is centered in a dark gray font.

The purification protocol takes approximately 45 minutes. When complete, the amplified DNA samples are in the processing plate located on Bravo deck position 7.

Stopping Point

If you do not continue to the next step, seal the sample wells and store at 4°C overnight or at -20°C for prolonged storage.

Library Quantity and Quality Assessment

Post-capture library analysis can be done using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol TS_D1000) and perform analysis on Agilent 4200 TapeStation. See [“Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape”](#) on page 59.
- Option 2: Prepare the analytical samples manually and perform analysis on Agilent 4200 or 4150 TapeStation or Agilent 5200 Fragment Analyzer. See [“Option 2: Analysis using an equivalent platform \(non-automated\)”](#) on page 63.

Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape

This section describes use of automation protocol TS_D1000 to prepare the D1000 assay sample plate by combining 2 μL of each DNA sample with 6 μL of D1000 sample buffer. Afterward, you transfer the prepared assay plate to the 4200 TapeStation instrument for analysis. For more information to do this step, see the [Agilent D1000 Assay Quick Guide for 4200 TapeStation System](#).

Allow the reagents used with either analysis system to equilibrate to room temperature for 30 minutes prior to use.

Prepare the workstation and Sample Buffer source plate

- 1 Clear the Labware MiniHub and BenchCel of plates and tip boxes.
- 2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
- 3 Turn off the ThermoCube device (see [page 17](#)) to restore position 9 of the Bravo deck to room temperature.
- 4 Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in [Setting the Temperature of Bravo Deck Heat Blocks](#). Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
- 5 Using the same processing plate (Eppendorf twin.tec or Armadillo) that was used for the 2X Amplification Master Mix source plate, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in [Table 45](#) to each well of column 3 of the plate. The final configuration of the Sample Buffer source plate is shown in [Figure 7](#).

Table 45 Preparation of the Sample Buffer Source Plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Processing Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 3 (A3-H3)	11.0 μL	17.0 μL	23.0 μL	29.0 μL	42.0 μL	80.0 μL

CAUTION

Make sure to add the D1000 Sample Buffer to column 3 of the source plate.

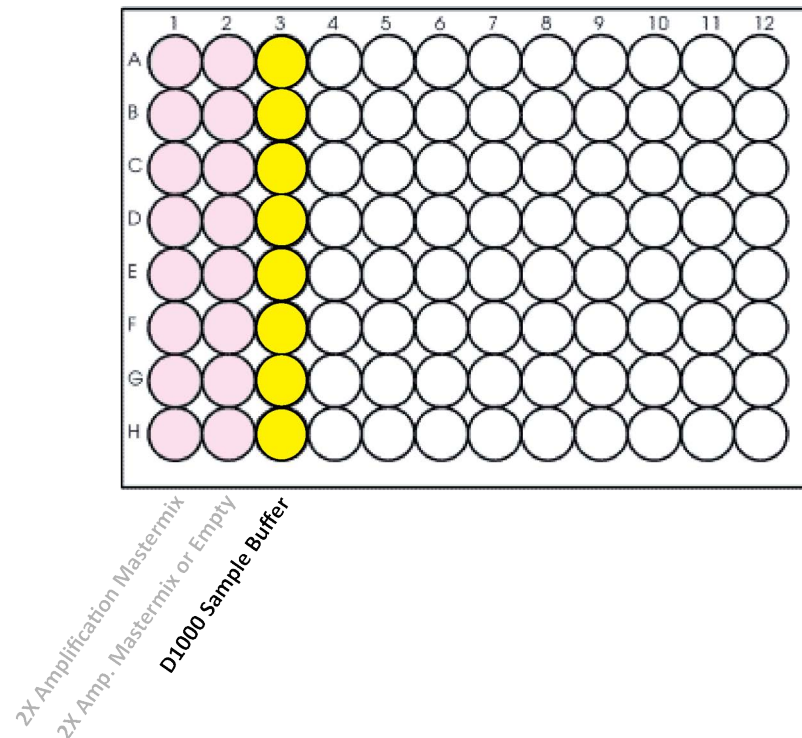


Figure 7 Configuration of the Sample Buffer source plate for protocol TS_D1000. Columns 1–2 were used in a previous protocol.

Load the Agilent NGS Workstation

- 6 Load the Labware MiniHub according to [Table 46](#), using the plate orientations shown in [Figure 4](#) on page 36.

Table 46 Initial MiniHub configuration for TS_D1000 protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

Library Purification, Quality Assessment, and Sample Pooling

Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape

7 Load the Bravo deck according to [Table 47](#).

Table 47 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified post-capture libraries in processing plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Sample Buffer source plate containing D1000 Sample Buffer in Column 3

CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument and the Agilent NGS Workstation, use only the specified Eppendorf twin.tec plates (Eppendorf p/n 951020401 or 951020619) for automated assay plate preparation.

The Agilent 2200 TapeStation system does not support use of the Eppendorf twin.tec plates. Do not load sample plates prepared using the automated protocol in the 2200 TapeStation instrument.

8 Load the BenchCel Microplate Handling Workstation according to [Table 48](#).

Table 48 Initial BenchCel configuration for TS_D1000 protocol

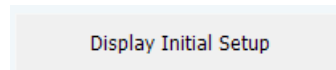
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_D1000

9 On the Avida DNA VWorks form, under **Select protocol to execute**, select the **TS_D1000** protocol.

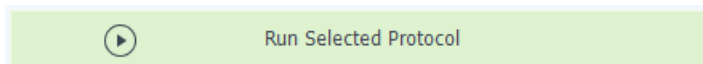
10 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

11 Click **Display Initial Setup**.



12 Verify that the NGS workstation has been set up as displayed on the right side of the form.

13 When verification is complete, click **Run Selected Protocol**.

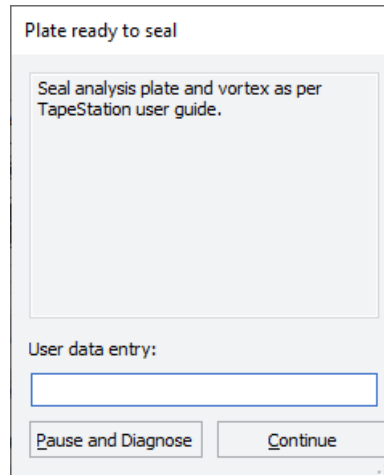


Library Purification, Quality Assessment, and Sample Pooling

Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape

Running the TS_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are pooled for sequencing on [page 65](#).

- 14** When prompted by VWorks as shown below, remove the Eppendorf twin.tec plate containing the analytical samples from position 6 of the Bravo deck, then click **Continue**. Seal the assay plate with a foil seal and vortex the sealed plate as directed in the [Agilent D1000 Assay Quick Guide for 4200 TapeStation System](#).



CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument, use only the specified 96-well plate foil seals (Agilent p/n 5067-5154).

For accurate quantitation, make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 1 minute, then spin briefly to collect the liquid.

Run the D1000 Assay and analyze the data

- 15** Load the analytical sample plate, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. Start the run.
- 16** Determine the concentration of each library by integrating under the entire peak.

See [Table 49](#) for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in [Figure 8](#) (cfDNA sample) and [Figure 9](#) (gDNA sample) to illustrate typical results.

Table 49 Library qualification guidelines

Input DNA type	Expected DNA fragment size peak position
cfDNA	320 bp (see Figure 8 for sample electropherogram)
Fragmented gDNA*	300–450 bp (see Figure 9 for sample electropherogram)

* Refers to high-quality gDNA samples. For gDNA derived from FFPE samples, the fragment sizes may be smaller.

Library Purification, Quality Assessment, and Sample Pooling

Option 2: Analysis using an equivalent platform (non-automated)

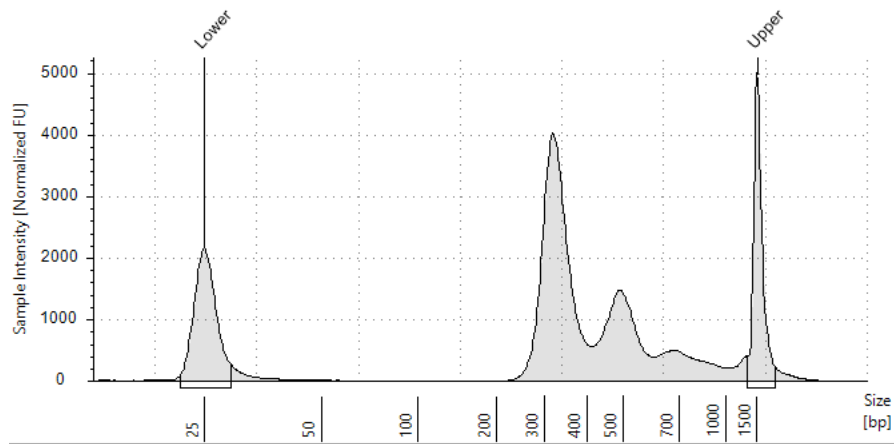


Figure 8 Library prepared from 10 ng of a cfDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using a D1000 ScreenTape assay.

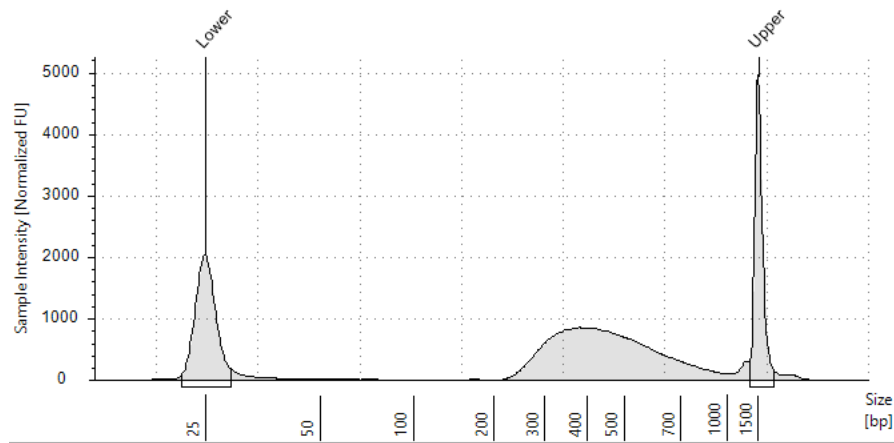


Figure 9 Library prepared from 10 ng of a high-quality, fragmented gDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using a D1000 ScreenTape assay.

Stopping Point If you do not continue to the next step, seal the plate and store at 4°C overnight or at –20°C for prolonged storage.

Option 2: Analysis using an equivalent platform (non-automated)

Using manual preparation of the analytical samples, you can analyze the DNA samples on an Agilent 5200 Fragment Analyzer or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see [Figure 8](#) through [Figure 9](#)). Verify that the electropherogram shows the expected DNA fragment size peak position (see [Table 49](#) for guidelines). [Table 50](#) includes links to assay instructions.

Table 50 Post-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	D1000 ScreenTape	Agilent D1000 Assay Quick Guide	1 μ L of sample mixed with 3 μ L of D1000 sample buffer
Agilent 5200, 5300, or 5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	Agilent NGS Fragment Kit (1-6000 bp) Guide	2 μ L of sample

Stopping Point If you do not continue to the next step, seal the sample wells and store at 4°C overnight or at -20°C for prolonged storage.

Sample Pooling for Multiplexed Sequencing (optional)

NOTE

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the two options described below. Option 2 can use the Aliquot_Captures automation protocol to pool samples.

Option 1: Pool samples at equal volumes (non-automated)

Dilute each indexed library to be pooled to the same final concentration (typically 4 nM to 15 nM, or the concentration of the most dilute sample) using 1X Low TE Buffer. This dilution step is performed by manually pipetting the 1X Low TE Buffer directly into the wells of the source plate. Then, combine equal volumes of all libraries to create the final pool in the destination plate.

Option 2: Pool samples at varying volumes (automated or non-automated)

Starting with indexed libraries at different concentrations, add the appropriate volume of each library to the destination well (either manually or using the Aliquot_Captures automation protocol) to achieve equimolar concentration in the pool. Then, adjust the pool to the desired final volume by adding the appropriate volume of 1X Low TE Buffer to each well. This volume adjustment is performed by manually pipetting the 1X Low TE Buffer directly into the wells of the destination plate. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where **V(f)** is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool (typically 4 nM–15 nM or the concentration of the most dilute sample)

is the number of indexes, and

C(i) is the initial concentration of each indexed sample

[Table 51](#) shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM DNA.

Library Purification, Quality Assessment, and Sample Pooling

Option 2: Pool samples at varying volumes (automated or non-automated)

Table 51 Example of volume calculation for total volume of 20 μL at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use (μL)
Sample 1	20 μL	20 nM	10 nM	4	2.5
Sample 2	20 μL	10 nM	10 nM	4	5
Sample 3	20 μL	17 nM	10 nM	4	2.9
Sample 4	20 μL	25 nM	10 nM	4	2
Low TE					7.6

Pool samples for multiplexed sequencing using automation (optional for Option 2)

The instructions below are for [Option 2: Pool samples at varying volumes \(automated or non-automated\)](#). Alternatively, you can perform this method using an entirely manual approach rather than with the Aliquot_Captures automation protocol.

- 1 Create a CSV (comma separated value) file with the headers shown in [Figure 10](#). The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in CSV format. The file must include rows for all 96 wells of the plate.
- 2 Enter the information requested in the header for each DNA sample. [Figure 10](#) shows an example spreadsheet.
 - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
 - In the SourceWell field, enter each well position on the source plate containing an amplified and captured indexed library that needs to be added to a pool. Use the Eppendorf twin.tec plate containing the purified indexed libraries as the source plate.
 - In the DestinationWell field, enter the well position on the destination plate for the pool.
 - In the Volume field, enter the volume (in μL) of each indexed library to be transferred from the source well to the destination well. The volume for each library is calculated from its concentration.
 - For all empty wells on the source plate, delete the corresponding rows in the CSV file.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	4.711292
3	abc	B1	A1	6.37105
4	abc	C1	A1	7.000448
5	abc	D1	A1	3.81144
6	abc	E1	A1	9.539072
7	abc	F1	A1	7.802747
8	abc	G1	A1	8.835171
9	abc	H1	A1	6.313131
10	abc	A2	A1	5.976286
11	abc	B2	A1	6.601183
12	abc	C2	A1	7.14449
13	abc	D2	A1	5.66431

Figure 10 Sample spreadsheet for option 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\Avida_DNA_v.B1.x.x.x\Aliquot Input File Templates\Aliquot_Captures_Template.csv** (where x.x.x is the version number).

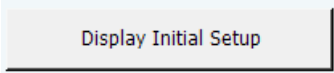
The Aliquot_Captures_template.csv file may be copied and used as a template for creating the CSV files for each Aliquot_Captures protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

- 3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\Avida_DNA_v.B1.x.x.x\Aliquot Input File Templates**.
- 4 Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
- 5 Load the Bravo deck according to [Table 52](#).

Table 52 Initial Bravo deck configuration for Aliquot_Captures protocol

Location	Content
5	Empty Agilent Deep Well plate
6	Empty tip box
8	New tip box
9	Purified amplified indexed libraries in processing plate

- 6 On the Avida DNA VWorks form, under **Select protocol to execute**, select the **Aliquot_Captures** protocol.
- 7 Click **Display Initial Setup**.



Display Initial Setup

- 8 Upload the CSV file created in [step 1](#) through [step 3](#) (page [66](#) to [67](#)).
 - a Click the “...” browse button below **Select Aliquot Input File** to open a directory browser window.



Select Aliquot Input File

- b Browse to the location where you saved the CSV file. Select the file and click **Open**.

The directory browser window closes, returning you to the Avida DNA setup form. The selected file location is listed in the field below **Select Aliquot Input File**.

- 9 Verify that the NGS workstation has been set up as displayed on the right side of the form.
- 10 When verification is complete, click **Run Selected Protocol**.



Run Selected Protocol

The aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the destination plate containing the library pools is on Bravo deck position 5.

Library Purification, Quality Assessment, and Sample Pooling**Option 2: Pool samples at varying volumes (automated or non-automated)**

- 11 Remove the destination plate from the Bravo deck.
- 12 Add the appropriate volume of 1X Low TE Buffer to each well to bring the pool to the necessary DNA concentration for sequencing.

8 Sequencing and NGS Analysis

Step 1. Prepare the sequencing samples [70](#)

Step 2. Sequence the libraries [70](#)

Step 3. Process and analyze the reads [71](#)

This chapter contains guidance on library sequencing and analysis. Refer to your specific Illumina sequencer's user guide for specific instructions on how to perform sequencing.

Step 1. Prepare the sequencing samples

The final Avida library pool is ready for sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform (Figure 11).

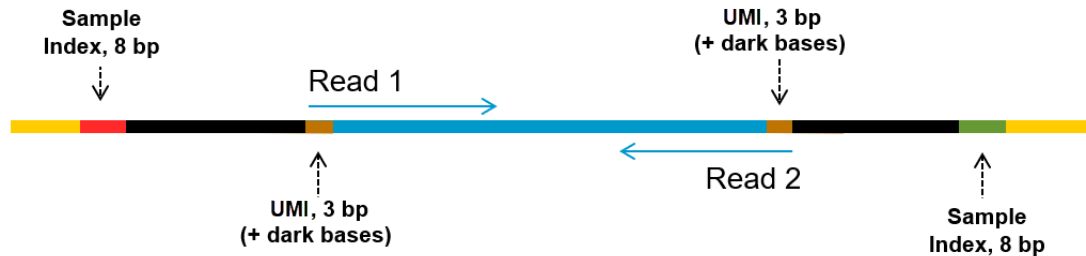


Figure 11 Content of Avida sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), unique dual sample indexes (red and green), duplex UMIs (brown), and the library PCR primers (yellow).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit and sequence the libraries using an Illumina instrument. Consult Illumina's documentation for kit configuration and seeding concentration guidelines.

Seeding concentration and cluster density may need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Step 2. Sequence the libraries

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument's software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate Cycles or Read Length value for your library read length and using 8-bp dual index reads. See Table 53 showing example settings for 2 × 150 bp sequencing.

Table 53 Run settings for 2x150 bp sequencing

Run Segment	Cycles/Read Length
Read 1	151*
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	151*

* Follow Illumina's recommendation to add one (1) extra cycle to the desired read length.

Follow Illumina's instructions for each platform and setup software option, incorporating the additional setup guidelines below:

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. See “[Online resource for Avida index sequences](#)” on page 10 for information on downloading the Avida index sequences spreadsheet.
- No custom primers are used for Avida library sequencing. Leave all Custom Primers options for Read 1, Read 2, Index 1 and Index 2 primers cleared/deselected during run setup.
- Turn off any adaptor trimming tools included in Illumina’s run setup and read processing software applications. Adaptors are trimmed in later processing steps to ensure proper processing of the adaptors, including the UMIs in the adaptor sequences.
- For runs set up using Illumina’s LRM, IEM, or BaseSpace applications, refer to Illumina’s instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the Avida index sequences provided online should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina’s specifications for each application. If you need assistance with Avida run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the Agilent Technical Support team (see [page 2](#)) or your local representative.

Step 3. Process and analyze the reads

Typical sequencing data processing steps are outlined below.

- 1 Generate demultiplexed FASTQ files for each sample using Illumina’s bcl2fastq, BCL Convert, or a similar software application. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes. Do not use the UMI trimming options offered in Illumina’s demultiplexing software.

NOTE

If your sequence analysis pipeline excludes UMIs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis.

If demultiplexing using bcl2fastq, UMIs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in [Table 53](#) on page 70). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, UMIs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in [Table 53](#) on page 70). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

Alternatively, the first 5 bases may be trimmed from the demultiplexed FASTQ files using a suitable processing tool (e.g., fgbio).

- 2 Use a suitable processing tool of your choice to trim and collect inline UMIs from each sequencing read. For example, UMI processing and sequence read alignment steps could be conducted with the fgbio best practice consensus pipeline, as described in: <https://github.com/fulcrumgenomics/fgbio/blob/main/docs/best-practice-consensus-pipeline.md>.

Sequencing and NGS Analysis

Step 3. Process and analyze the reads

The output includes deduplicated reads in BAM format with, 1) single-stranded UMI consensus reads, and 2) double-stranded UMI consensus reads.

- Inline UMIs are added to both ends of the DNA inserts in the assay. To collect the UMIs and process them for analysis, one approach is to trim off 5 bases at the beginning of each read, take the first 3 of the 5 bases as UMI, and discard the remaining 2.
- 3** Perform variant calling and filtering using the BAM files generated in [step 2](#) above.
 - 4** Collect QC metrics on alignment.
 - 5** (Optional) Generate a report containing the QC metrics and variant calls.

9

Reference

Reagent Kit Contents	74
Avida Index Primer Pair Information	75
Avida Custom AddOn Panels	77
Troubleshooting Guide	78

This section contains reference information, including Reagent Kit contents, index primer pair information, and troubleshooting tips.

Reagent Kit Contents

[Table 1](#) on page 10 lists the Avida DNA reagent kits and their sub-kit boxes. Detailed contents of those sub-kit boxes are shown in [Table 54](#) through [Table 56](#).

Avida Sub-Kit Details

Table 54 Avida DNA Reagent Box 1

Kit Component	96 Reaction Kit (p/n 5282-0140)
End Prep Buffer	tube with purple cap
End Prep Enzyme	tube with blue cap
Ligation Buffer	bottle
Ligation Enzyme	tube with yellow cap
Adaptor for ILM	tube with orange cap
Hyb Blocker	tube with red cap
Hyb Buffer	bottle
Hyb Enhancer	amber tube with green cap
2X Amplification Mastermix	bottle

Table 55 Avida DNA and Duo Reagent Box 2

Kit Component	96 Reaction Kit (p/n 5282-0142)
Library Wash Buffer	bottle
Hyb Wash Buffer 1	bottle
Hyb Wash Buffer 2	bottle
Resuspension Buffer	bottle
Nuclease-Free Water	bottle

Table 56 Avida Beads Box

Kit Component	96 Reaction Kit (p/n 5282-0144)
Library Binding Beads	bottle
Capture Beads	tube with amber cap

Avida Index Primer Pair Information

The Avida Index Primer Pairs are provided pre-combined in the well of either a strip tube or a plate (see [Table 57](#)). Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. One primer pair (forward and reverse primers) is provided as a single-use aliquot in each well of an 8-well strip tubes or 96-well plate.

See [“Online resource for Avida index sequences”](#) on page 10 for information on downloading the Avida index sequences spreadsheet.

Table 57 Avida Index Primer Pairs for ILM Content

Kit Component	96 Reaction Kit Format
Avida Index Primer Pairs for ILM	Clear 96-well plate with index pairs 1–96 -- OR -- Blue 96-well plate with index pairs 97–192

Index Primer Pair Strip Tube and Plate Maps

[Table 58](#) and [Table 59](#) show the plate positions of the Avida Index Primer Pairs provided with 96 reaction kits.

Table 58 Plate map for Avida Index Primer Pairs 1-96, provided in clear plate

	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

9 Reference

Index Primer Pair Strip Tube and Plate Maps

Table 59 Plate map for Avida Index Primer Pairs 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Avida Custom AddOn Panels

When including an Avida DNA Custom AddOn panel in the hybridization, make the protocol adjustments described below.

CAUTION

Make sure that the Custom AddOn panel is compatible with the Avida catalog panel you are using in the workflow. If needed, check with Agilent [Technical Support](#) or your local sales representative for design ID compatibility. Custom AddOn panels are limited to 250 kb in size.

During preparation of the Hyb master mix on [page 33](#), substitute the reagents and volumes in [Table 20](#) or [Table 21](#) with those shown in [Table 60](#) or [Table 61](#) below (depending on panel size).

If using the Avida DNA Focused Cancer Panel (design ID D3483231) as the Avida DNA catalog panel, make sure that the Custom AddOn panel is <20 kb. Consider designing a Custom Avida DNA Panel if an add-on larger than 20 kb is required.

Table 60 Preparation of Hyb master mix using Avida DNA Custom AddOn Panel – Total panel size <500 kb

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water (bottle)	13.5 µL	172 µL	300 µL	428 µL	554 µL	810 µL	1570 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	102 µL	150 µL	290 µL
Hyb Enhancer (amber tube with green cap)	6 µL	76 µL	134 µL	190 µL	246 µL	360 µL	698 µL
Avida DNA panel (catalog)	4 µL	50 µL	90 µL	126 µL	164 µL	240 µL	466 µL
Avida DNA Custom AddOn Panel	4 µL	50 µL	90 µL	126 µL	164 µL	240 µL	466 µL
Total Volume	30 µL	380 µL	670 µL	950 µL	1230 µL	1800 µL	3490 µL

Table 61 Preparation of Hyb master mix using Avida DNA Custom AddOn Panel – Total panel size ≥500 kb

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water (bottle)	13.5 µL	172 µL	302 µL	430 µL	558 µL	814 µL	1578 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	104 µL	150 µL	292 µL
Hyb Enhancer (amber tube with green cap)	5 µL	64 µL	112 µL	158 µL	206 µL	302 µL	584 µL
Avida DNA panel (catalog)	4 µL	52 µL	90 µL	126 µL	166 µL	242 µL	468 µL
Avida DNA Custom AddOn Panel	4 µL	52 µL	90 µL	126 µL	166 µL	242 µL	468 µL
Total Volume	29 µL	370 µL	650 µL	920 µL	1200 µL	1750 µL	3390 µL

Troubleshooting Guide

If yield of libraries is lower than previous runs

- Make sure that the Library Binding Beads (used in [“Step 7. Prepare the Library Prep master mix reagent source plate”](#) on page 34) have equilibrated to room temperature before use.
- During [“Step 6. Perform PCR cycling”](#) on page 53, the number of cycles used in for Amplification stage 2 may require optimization. Refer to the [Note on page 54](#) for guidelines.

If Library Binding Beads settle while sitting in the Library Prep master mix reagent source plate

- Before setting up the plate, allow the stock bottle of Library Binding Beads to equilibrate to room temperature and make sure the bottle is thoroughly vortexed.
- Avoid leaving the Library Prep master mix reagent source plate on ice for longer than necessary before starting the Library Prep runset.

If any of the reagents in the Library Prep master mix reagent source plate run out

- Make sure that the correct reagent volumes are added to the appropriate wells of the plate. Refer to [Table 22 on page 34](#) and [Figure 3](#) on page 35.
- Briefly centrifuge the plate at 50 × g for 1 second to drive the well contents off the walls.

If peak positions in the electropherogram are not as expected

- The indexing PCR reactions may have been contaminated with amplicon from another assay. Maintain separate work areas for pre-PCR and post-PCR steps. If maintaining separate areas is not an option, clean the at-risk surfaces with 10% bleach. Review [“Important practices for preventing contamination”](#) on page 8.

If low uniformity of coverage with high AT-dropout is observed in library sequencing results

- Make sure that the hybridization temperature used in the hybridization thermal cycle program is 60°C as directed in [Table 26](#) on page 39.

In This Book

This guide contains information to run the Avida DNA workflow using automation protocols provided with the Agilent NGS Workstation Option B.

