

Bravo Seahorse Assay Workbench User Guide

Introduction

Cell-based assays often require several liquid handling steps, which can influence data quality. Lack of consistent sample preparation can result in unacceptable variability across wells, plates, or replicate assays. Such pipetting steps can also be labor-intensive, especially when performing multiplexed or replicate assays in large numbers, as is often required in drug discovery projects.

To address these issues when performing Agilent Seahorse XFe96 assays, several key manual pipetting steps can be automated using the Agilent Bravo automated liquid handling platform with the Bravo Seahorse Assay Workbench software. Performing liquid handling steps using the Bravo platform provides value for the researcher with respect to data quality and reduction in assay preparation (hands-on) time. Both benefits allow for increased productivity due to more consistent data as well as increased walk-away time during the XF assay.

Seahorse XF assays have common sample preparation elements regardless of the specific application or kit being used:

1. Washing cells (exchanging growth media for assay media)
2. Compound Preparation (dilutions)
3. Compound Port Loading into the XFe96 Sensor Cartridge

These workflow steps are routine, involve substantial pipetting, and have the potential to add error to assays; as such, they are good candidates for automation.

Required equipment and application software

- Agilent Seahorse XFe96 analyzer
- Agilent Bravo automated liquid handler configured for use with Agilent Seahorse XFe96 consumables (through the Bravo Seahorse assay workbench)

General guidelines

The protocols described in this User Guide are designed to work with the Agilent Seahorse XF Bravo deck configuration. An installer for the Bravo Seahorse Assay Workbench software can be acquired by contacting a Bravo Application Engineer. Contact the Automation Technical Support Team at service.automation@agilent.com or 1-800-979-4811.

Ensure that the labware is placed on the deck exactly as shown on the form. Failure to place labware in the appropriate deck positions could result in a crash, potentially resulting in damage to the Bravo liquid handling head. Labware needs to be properly seated on each deck position, and the MTC controller should be turned on for temperature control.

Required labware and consumables

Table 1. Agilent Bravo labware and XF consumables required for cell washing, compound dilution, and XFe96 cartridge port loading.

Agilent Labware and XF Consumables	Part Number	Quantity Required per XFe96 Assay		
		Cell Washing	Compound Dilution	Cartridge Loading
24-Column Reservoir (Agilent reservoir, 24 column, polypropylene, 3.25 mL/column, pyramid base geometries)	201296-100	-	-	1
96-Well Storage/Reaction Plate (Agilent storage/reaction microplate, 96-well, ultrahigh purity polypropylene, 1 mL/square well, conical bottoms)	201276-100	-	2	-
Reservoir (Agilent reservoir, single cavity, polypropylene, 86 mL, 96 pyramids base geometry)	201254-100	2	1	-
96 V11 LT250 Tip Box (full)	19477-002	1	2	1
96 V11 LT250 Tip Box (empty)		-	1	1
Agilent Seahorse XFe96 FluxPak ^a	102416-100	1 cell plate (96 Seahorse XFe96 cell plate)	1 XFe96 cartridge with utility plate (96 Seahorse XFe96 cartridge with utility plate, 384 Ports)	
Agilent Seahorse XF assay media ^b	Assay dependent	90 mL	40 mL	20 mL
Seahorse XF glucose (1.0 M solution) ^b	103577-100	XF assay dependent		
Seahorse XF pyruvate (100 mM solution) ^b	103578-100	XF assay dependent		
Seahorse XF L-glutamine (200 mM solution) ^b	103579-100	XF assay dependent		
Seahorse XF assay kit	-	Application dependent		

^a A FluxPak includes 18 XFe96 sensor cartridges, 18 utility plates, 20 cell culture microplates, and 500 mL of XF calibrant.

^b Choice of XF media used to prepare XF assay media will be XF kit/assay dependent.

Seahorse XF assay preparation and setup

Figure 1 presents a simplified workflow for XF assay setup.

Prepare the XF assay cell plate, sensor cartridge, XF assay media, and XF kit reagents according to the recommended standard procedures.

See the XF Assay Learning Center for all details pertaining to preparation for XFe96 assay materials, including:

- Seeding and growth of cells in XF96 cell culture microplates
- Hydration of XFe96 sensor cartridges
- Preparation of appropriate XF assay media
- Preparation of XF kit sensor cartridge injection solutions

Using the Bravo Seahorse Assay Workbench

1. Open the Bravo Seahorse Assay Workbench.
2. **Select the desired protocol:** Cell Plate Wash (media exchange), Serial Compound Dilution and Port A Loading, Simple Cartridge Port Loading A–D (1 to 4 ports), or Simple Cartridge Port Loading B–D (1 to 3 ports), see Figure 2A.

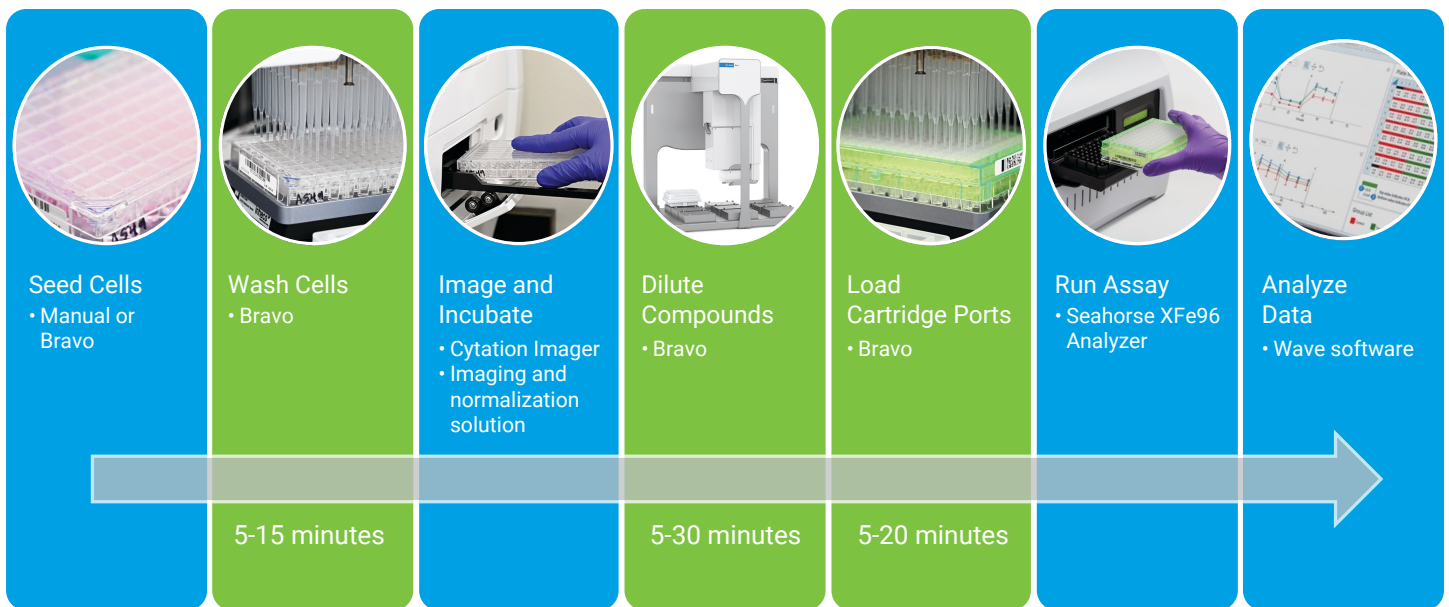


Figure 1. Agilent Seahorse XF assay setup using the Bravo automated liquid handling platform.

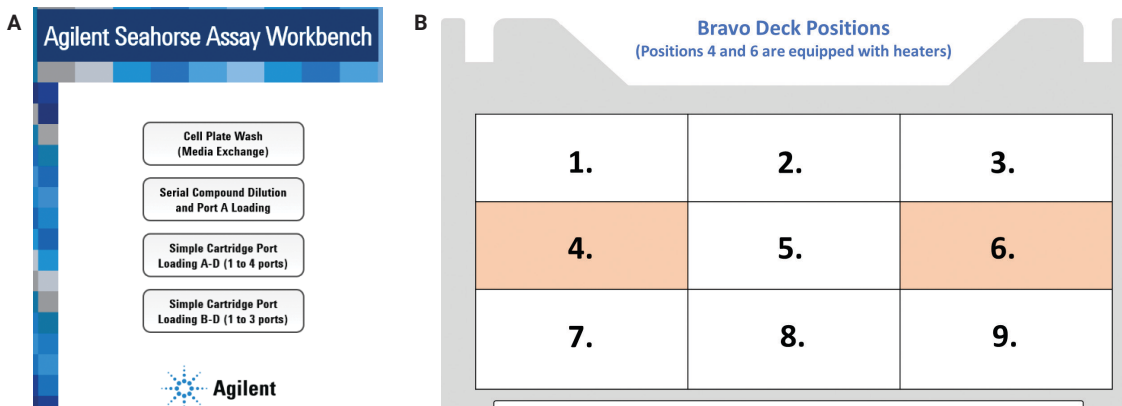


Figure 2. A) Bravo Seahorse Assay Workbench main menu; B) Bravo deck positions.

- Follow the directions described in the form.
 - Set up the Bravo deck with the proper consumables on the appropriate deck position (1–9, Figure 2B), which will depend on the selected procedure.
 - Ensure that the MTC controller for heaters in deck positions 4 and 6 is turned on.

- Start the protocol.

The protocol will immediately begin when **Start Protocol** is selected. Follow any prompts that appear, for example, modifying the tip state or adding assay media to the appropriate deck location. Once all prompts are satisfied, heaters on positions 4 or 6 will warm to 37 °C. This can take several minutes if the heaters were off. Once the deck positions reach 37 °C, the protocol will be executed.

Modifying the tip state

Some protocols require user input to identify the contents of tip boxes (empty or full). Ensure that the tip boxes are placed on the deck according to the layout before starting the protocol. To modify the tip state, start the protocol, and when prompted:

- PAUSE** the protocol from the dialog box
- Select **TIP STATE EDITOR** from the menu on the protocol form
- Select the tip box position to be edited from the Tip State Editor
- Click on the three dots that appear on the right to open the **Tip Position Dialog box**
- Assign tips according to the form and press “OK”
- Close the Tip State Editor
- CONTINUE** the protocol from the dialog box

Protocols

1. Cell plate wash (media exchange)

What the protocol will do:

Wash a single XF96 cell culture microplate plate. Remove lid; multiple exchanges (2 to 3) of XF assay media replace the original cell growth media; return lid. The system uses gentle aspirate and dispense speeds to minimize potential cell loss. The XF cell culture microplate is maintained at 37 °C throughout the protocol, and kept at 37 °C until the protocol complete dialog box is acknowledged.

This protocol requires that cells have been seeded in 80 µL of growth media, as recommended for XF96 cell culture microplates. The final volume in the well after the wash is 180 µL.

Preparation for running the protocol:

- Gather labware and consumables listed in Table 1 for **Cell Wash**.
- Prepare XF assay media with supplements according to the XF assay being performed, and warm to 37 °C. A minimum of 80 mL is required to wash a cell plate. The reservoir has a capacity of 86 mL.

Running the protocol:

- Open the Bravo Seahorse Assay Workbench, and select **Cell Plate Wash**. Follow the directions on the screen, and arrange the deck according to the layout on the right side of the screen, shown in Figure 3, and described below.
- Upon completion of cell plate wash protocol, place the XF96 cell plate in a non-CO₂ incubator.

To set up the Bravo deck (Figure 3)

- XF96 cell plate:** Place a microplate containing cells seeded WITH LID on deck position 6.
- Assay media:** Add 80 mL of 37 °C XF assay media to an Agilent 96 pyramid base reservoir plate, then place on deck in position 9.
- Full tip box:** Place a full box (96 V11 LT 250 Tip Box) on deck position 7.
- Empty Agilent 96 pyramid base reservoir plate:** Place on deck position 8.
- Empty Bravo deck positions (no labware/consumables):** Positions 1 to 5.

The screenshot shows the 'Cell Plate Wash (Media Exchange)' protocol screen. It includes an 'Instructions' section with three steps: 1. Set up the Bravo Deck, 2. Fill the Reservoir, and 3. Click the Start Run button. A 'Deck Layout' diagram shows a 9-position deck with specific labware assignments: 1-5 are empty, 6 is the Cell Plate with Lid, 7 is the Tip box, 8 is the Reservoir for waste media, and 9 is the Reservoir for new media. A 'Labware Table' lists the items for each deck location, including the 96 Seahorse XF96 Cell Plate, 96 V11 LT 250 Tip Box, and two Agilent 96 pyramid base reservoir plates.

Deck Location	Labware Type
1	Empty (No Labware)
2	Empty (No Labware)
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	96 Seahorse XF96 Cell Plate (P/N 102416-100)
7	96 V11 LT 250 Tip Box (P/N 19477.002)
8	Reservoir, Agilent, PP, 86mL, 96 pyramid base (P/N 201254-100)
9	Reservoir, Agilent, PP, 86mL, 96 pyramid base (P/N 201254-100)

Figure 3. Cell Plate Wash (Media Exchange) screen. Bravo instructions, deck layout, and labware table as seen in the Bravo Seahorse Assay Workbench for cell plate washing.

2. Simple cartridge port loading A-D (1 to 4 ports)

What the protocol will do:

Aspirate reagents from 24 column reservoir and dispense injection solutions into 1, 2, 3, or 4 ports in the XFe96 sensor cartridge, in the order of A, B, C, and D, respectively. Port loading volumes are: A = 20 μ L, B = 22 μ L, C = 25 μ L, and D = 28 μ L. Each group of ports (A, B, C, or D) will receive the same injection solution in the respective port, and is intended to be used with standard XF assay workflows with an initial well volume of 180 μ L. The XFe96 cartridge is maintained at 37 °C throughout the protocol, and will remain at 37 °C until the **protocol complete** dialog box is acknowledged.

This protocol may be used for the following XF assay kits and applications (Table 2), as well as custom-designed assays using 1 to 4 ports. In this protocol, all wells receive the same compound in each port. This design would be implemented when the intervention or variable is a medium-to-long-term compound exposure, genetic modification, or comparison of different cell types or treatments made before the XF assay.

Please see Section 4 (Port loading B–D) if the assay design calls for a comparison of different compounds/acute treatments to be delivered to the cells through port A.

Table 2. A List of common XF assay kits that may be automated with the Bravo platform when using the **Simple Cartridge Port Loading A-D (1 to 4 ports)** protocol.

XF Kit or Application		Ports Used	Compounds Used
Seahorse XF Cell Mito Stress Test		A > B > C	Oligo > FCCP > Rtn/AA
Seahorse XF Real-Time ATP Rate Assay		A > B	Oligo > Rtn/AA
Seahorse XF Glycolytic Rate Assay		A > B	Rtn/AA > 2-DG
Seahorse XF Cell Energy Phenotype Assay		A	Oligo + FCCP
Seahorse XF Glycolysis Stress Test		A > B > C	Glucose, Oligo, 2-DG
Seahorse XF PMP and Isolated Mitochondria Assays	Coupling	A > B > C	Oligo, FCCP, AA
	Electron Flow	A > B > C > D	CI substrate* > Rtn > Succinate > Ascorbate/TMPD

Preparation for running the protocol:

- Gather labware and consumables listed in Table 1 for **Cartridge Loading**.
- Prepare assay media with supplements according to the XF assay being performed, and warm to 37 °C. A minimum of 20 mL is required to prepare injection compounds.
- Prepare injection solutions according to the XF assay being performed. Injection solutions may be first prepared, then transferred into the 24-column reservoir or prepared directly in the 24-column reservoir. A 3-mL volume of each injection solution is required to ensure sufficient volume for all 96 ports (Figure 4).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
P O R T A		P O R T B		P O R T C		P O R T D																	

Figure 4. 24-column reservoir injection solution (port compound) layout for 4-port loading protocol (A > B > C > D). Add 3.0 mL injection solution per column. Place Port A compound in Column 1, Port B compound in column 3, Port C in column 5, and Port D in column 7.

Running the protocol

1. Open the Bravo Seahorse Assay Workbench, and select **Simple Cartridge Port Loading A-D (1 to 4 ports)**.
2. Follow the directions on the left side of the screen, and arrange the deck according to the layout shown in Figure 5, and described below.
3. Upon completion of the Bravo protocol, begin the appropriate XF assay, and manually insert the cartridge into the XF instrument for calibration when prompted.

To set up the Bravo Deck (Figure 5)

- **XFe96 sensor cartridge:** Place a cartridge with its utility plate and a lid on deck position 4.
- **Injection solutions:** Place the 24-column reservoir containing injection solutions in position 5.
- **Full tip box:** Place a full box (96 V11 LT 250 Tip Box) on deck position 2.
- **Empty tip box:** Place an empty tip box (96 V11 LT 250 Tip Box) on deck position 1.

3. Serial compound dilution and port A loading

What the protocol will do

A specialized protocol for generating eight individual two-fold serial dilution series. Stock concentrations of each compound are manually loaded into a storage plate in column 1. Each library compound to be diluted is preloaded as a stock into a separate storage plate (the source plate). The Bravo then executes a 10-step two-fold serial dilution of all eight compounds, including distribution of diluent, followed by loading port A of the cartridge with 20 μ L of all dilutions. Figure 6 illustrates the resulting XF assay plate layout.

Simple Cartridge Port Loading A-D (1 to 4 ports)

Instructions

Step	Instruction
1	Set up the Bravo Deck so that it matches the Bravo Deck Setup and Labware Table sections shown on the right.
2	Select ports to load on 96 Seahorse XFe96 Cartridge w/ Utility Plate, 384 Ports . If Port A is being used for a pretreatment of more than one condition or with the simple serial dilution method, use 'Simple Cartridge Port Loading B-D' program.
3	Transfer injection solutions into 24 column reservoir (Port A in col 1, Port B in col 3, Port C in col 5, and Port D in col 7).
4	Click the Start Run button to start the cartridge port loading.
5	When prompted to setup the tip state editor, configure an empty tip box on deck location 1 and a full tip box with new tips on deck location 2.

Deck Layout

1. Tip box (250 μ L Tips) - EMPTY	2. Tip box (250 μ L Tips) - FULL	3. EMPTY (no labware)
4. XFe96 Cartridge w/ Utility Plate, 384 Ports with Lid	5. 24 Column Reservoir	6. EMPTY (no labware)
7. EMPTY (no labware)	8. EMPTY (no labware)	9. EMPTY (no labware)

Labware Table

Deck Location	Labware Type
1	96 V11 LT 250 Tip Box (P/N 19477.002)
2	96 V11 LT 250 Tip Box (P/N 19477.002)
3	Empty (No Labware)
4	96 Seahorse XFe96 Cartridge w/ Utility Plate, 384 Ports (P/N 102416-100)
5	24 Col Reservoir, Agilent, PP, pyramid base, 3.25 mL/col (P/N 201296-100)
6	Empty (No Labware)
7	Empty (No Labware)
8	Empty (No Labware)
9	Empty (No Labware)

Select Cartridge Port

Port	Volume(μ L)
<input checked="" type="checkbox"/> A	20
<input type="checkbox"/> B	22
<input type="checkbox"/> C	25
<input type="checkbox"/> D	28

Figure 5. Simple Cartridge Port Loading A–D screen. Bravo instructions, deck layout, and labware table as seen in the Bravo Seahorse Assay Workbench for loading 1, 2, 3, or 4 injection ports.

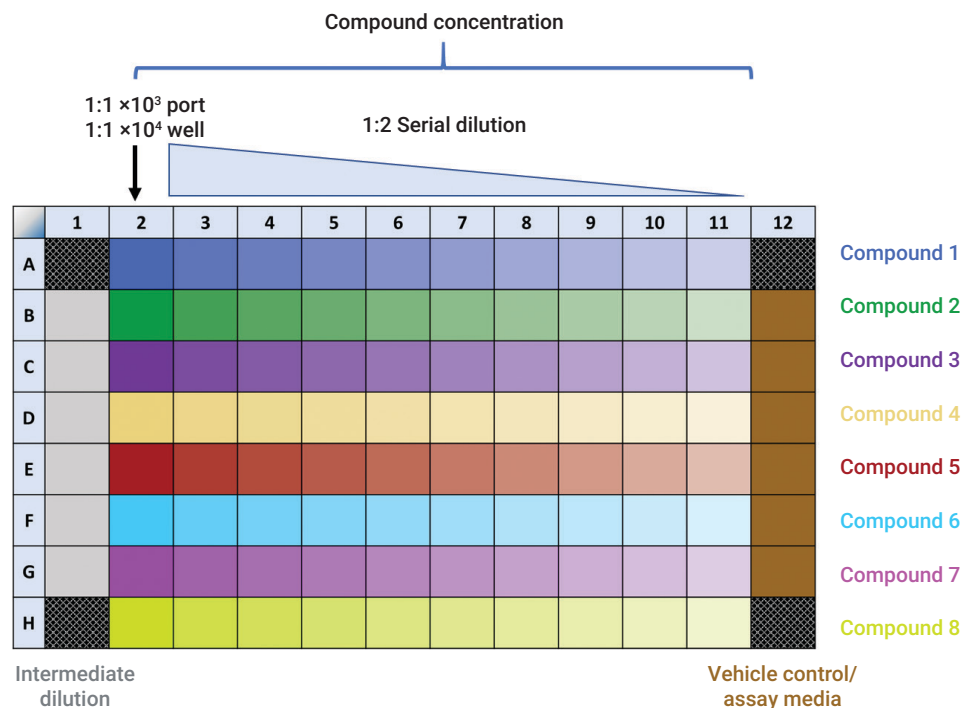


Figure 6. Resulting XF assay layout when using the serial compound dilution and port A loading protocol. See Note next page.

The top concentration in the dilution series, located in column 2, is a 1:1,000 dilution of the stock solution. This translates to 1:10,000 post injection. For example, a 10 mM DMSO stock will generate a dilution series from 10 to 0.02 μM (1 to 0.002 μM post injection). The XF sensor cartridge is maintained at 37 °C throughout the protocol, and will remain at 37 °C until the **protocol complete** dialog box is acknowledged.

Note: By default, solutions from columns 1 (intermediate dilutions of library compounds) and 12 (XF assay media/vehicle control) of the compound dilution plate (deck position 5) will be loaded into the A ports of columns 1 and 12 of the XFe96 cartridge, respectively. If these columns are required for other uses, tips in columns 1 and 12 from the tip box on deck position 8 may be removed before running the protocol, and port A loaded independently for those wells.

This protocol may be followed with the **Simple Cartridge Port Loading B-D (1 to 3 ports)** to load additional assay injection solutions (for example, XF Cell Mito Stress Test Compounds), described in Section 4.

Preparation for running the protocol

1. Gather the labware and consumables listed in Table 1 for **Cartridge Loading**.
2. Prepare assay media with supplements according to the assay being performed, and warm to 37 °C.
3. Transfer aliquots of stock test compounds to wells A1 through H1 in column 1 of a 96-well storage/reaction plate. A minimum volume of 5 μL is recommended for each compound.

Running the protocol

1. Open the Agilent Seahorse Assay Workbench, and select **Serial Dilution and Port A Loading**.
2. Follow the directions on the left side of the screen, and arrange the deck according to the layout shown in Figure 7, and described below.
3. Upon completion of the Bravo protocol, begin the appropriate XF assay, and manually load the cartridge into the XF instrument for calibration when prompted.

To set up the Bravo deck (Figure 7)

- **XFe96 sensor cartridge:** Place a cartridge with a lid on deck position 4.
- **Dilution plate:** Place an empty storage plate (Agilent 96-well, polypropylene, 1 mL/square well, conical bottom) on deck position 5.
- **Library source plate:** Place the source plate, a storage plate (Agilent, 96-well, polypropylene, 1 mL/square well, conical bottoms) containing at least 5 μL of each stock library compound (C1 to C8 in plate column 1, Figure 8) onto deck position 6.

The screenshot shows the 'Serial Compound Dilution and Port A Loading' screen. It includes an 'Instructions' section with 5 steps, a 'Deck Layout' diagram showing the arrangement of labware on the deck, and a 'Labware Table' listing the labware types and part numbers for each deck location.

Deck Location	Labware Type
1	96 V11 LT 250 Tip Box (P/N 19477.002)
2	96 V11 LT 250 Tip Box (P/N 19477.002)
3	Empty (No Labware)
4	96 Seahorse XFe96 Cartridge w/ Utility Plate, 384 Ports (P/N 102416-100)
5	96 Agilent, PP, 1.1mL sq well, v btm (P/N 201276-100)
6	96 Agilent, PP, 1.1mL sq well, v btm (P/N 201276-100)
7	Reservoir, Agilent, PP, 86mL, 96 pyramid base (P/N 201254-100)
8	96 V11 LT 250 Tip Box (P/N 19477.002)
9	Empty (No Labware)

Figure 7. Serial Compound Dilution and Port A Loading screen. Bravo instructions, deck layout, and labware table as seen in the Bravo Seahorse Assay Workbench for serial dilution of compounds and loading port A with up to eight individual compound dilution series.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1											
B	C2											
C	C3											
D	C4											
E	C5											
F	C6											
G	C7											
H	C8											

Figure 8. Setup for Compound Library Source plate. Place 5 μL of each compound to be diluted a well in column 1 of a storage plate.

- **Reservoir for new media:** Place a reservoir (Agilent reservoir, single cavity, polypropylene, 86 mL, 96 pyramids base geometry) with 86 mL of appropriate XF assay media (dependent on XF assay performed) on deck position 7.
- **Full tip boxes:** Place full boxes (96 V11 LT 250 Tip Box) on deck positions 2 and 8.
- **Empty tip box:** Place an empty box (96 V11 LT 250 Tip Box) on deck position 1.

4. Simple Cartridge Port Loading B-D (1 to 3 ports)

What the protocol will do

Load injection solutions into up to three ports in the XF sensor cartridge in the order: B, C, D*. Each port will receive the same solution in a fixed volume intended to be used with a standard XF assay workflow that assumes an initial well volume of 180 μ L. Port volumes are B = 22 μ L, C = 25 μ L, and D = 28 μ L. The XFe96 cartridge is maintained at 37 °C throughout the protocol, and will remain at 37 °C until the protocol complete dialog box is acknowledged.

Note: This protocol assumes that port A has been loaded independently with 20 μ L of an appropriate injection solution (assay dependent), and is most often used:

- After preparing a serial dilution series of several test compounds (see protocol *Serial Compound Dilution and Port A Loading*).
- After loading port A with different compounds/cell treatments (for example, compound library).

This protocol may be used for the following XF assay kits and applications (Table 3), as well as custom-designed assays.

Table 3. A list of XF assay kits and applications that may be automated with the Bravo platform when using the **Simple Cartridge Port Loading B-D (3 to 4 ports)** protocol.

XF Kit or Application*		Ports Used*	Compounds Used*
Seahorse XF Cell Mito Stress Test (Acute Injection)		B > C > D	Oligo > FCCP > Rtn/AA
Seahorse XF Real-Time ATP Rate Assay (Induced)		B > C	Oligo > Rtn/AA
Seahorse XF Glycolytic Rate Assay (Induced)		B > C	Rtn/AA > 2-DG
Seahorse XF Glycolysis Stress Test (Acute Injection)		B > C > D	Glucose > Oligo > 2-DG
Seahorse XF PMP and Isolated Mitochondria Assays	Coupling	B > C > D	Oligo, FCCP, AA

*This protocol assumes port A has been loaded independently with 20 μ L of an appropriate injection solution (assay dependent).

Preparation for running the protocol

- Gather labware and consumables listed in Table 1 for **Cartridge Loading**.
- Prepare assay media with supplements according to the XF assay being performed, and warm to 37 °C. A minimum of 20 mL is required to prepare injection compounds.

- Prepare injection solutions according to the assay being performed. Injection solutions may be prepared, then transferred into the 24-column reservoir or prepared directly in the reservoir. Three milliliters of each injection solution is required to ensure sufficient volume for all 96 ports (Figure 9).
- * **Note:** This protocol does not load port A. It is essential that compounds are transferred to/prepared in the specified location in the reservoir (Figure 9) to ensure that the correct compound is loaded in the corresponding port.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
E M P T Y		P O R T B		P O R T C		P O R T D																	

Figure 9. 24-Column reservoir injection solution (port compound) layout for a three-port loading protocol. Place 3 mL of Port B compound in column 3, Port C in column 5, and Port D in column 7.

Running the protocol

1. Open the Agilent Seahorse Assay Workbench, and select **Simple Cartridge Port Loading B-D (1 to 3 ports)**.
2. Follow the directions on the left side of the screen, and arrange the deck according to the layout shown in Figure 10, and described below.
3. Upon completion of the Bravo protocol, begin the appropriate XF assay, and manually load the cartridge into the XF instrument for calibration when prompted.

To set up the Bravo deck (Figure 10)

- **XFe96 sensor cartridge:** Place the cartridge with its utility plate and a lid on deck position 4.
- **Injection solutions:** Place the 24-column reservoir containing injection solutions in position 5.
- **Full tip box:** Place a full box (96 V11 LT 250 tip box) on deck position 2.
- **Empty tip box:** Place an empty box (96 V11 LT 250 Tip Box) on deck position 1.
- **Empty deck position (no labware/consumables):** Positions 3 and 6 to 9

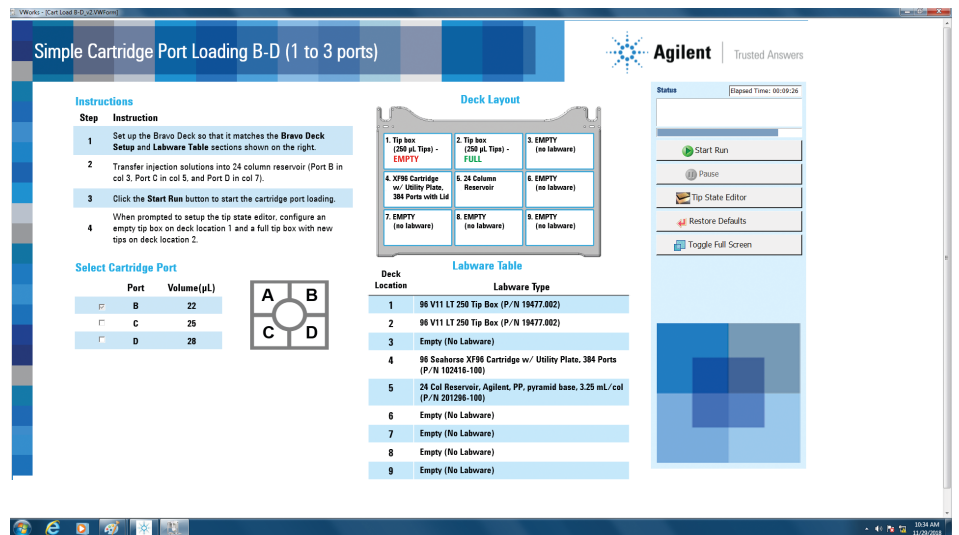


Figure 10. Simple Cartridge Port Loading B-D screen. Bravo instructions, deck layout, and labware table as seen in the Bravo Seahorse Assay Workbench for loading one, two, or three injection ports.

Resources

1. Maximizing efficiency and performance of Seahorse XF assays via automation
2. Seahorse XF Assay Learning Center
3. Seeding Adherent Cells in Agilent Seahorse XF96 Cell Culture Microplates
4. Washing Adherent Cells in Agilent Seahorse XF96 Cell Culture Microplates
5. Loading the Agilent Seahorse XFe96 Sensor Cartridge Injection Ports
6. Agilent Bravo Liquid Handling Support Information

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