



A part of **Agilent Technologies**

XF^e96 Training Manual



Instrument Serial #

XF^e96 Analyzer Quick Info

XF^e96 Analyzer Consumables

XF Stress Test Kits & Reagents

Part #	Description	# of Microplates
103015-100	XF Cell Mito Stress Test Kit	6
103020-100	XF Glycolysis Stress Test Kit	6
102504-100	XF Plasma Membrane Permeabilizer	6
102720-100	XF Palmitate-BSA FAO Substrate	3

XF FluxPaks

Part #	Description	# of Cartridges
102416-100	XF ^e 96 FluxPak	18
102601-100	XF ^e 96 FluxPak mini	6

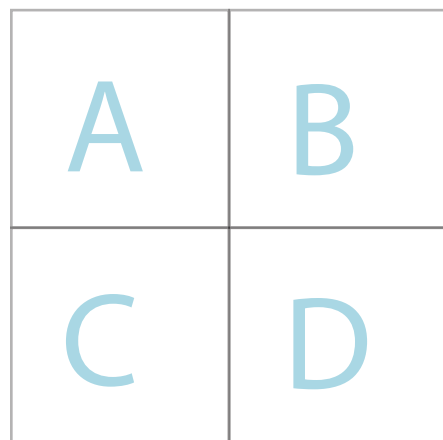
XF Microplates

Part #	Description	# of Microplates
101085-004	XF96 V3 PS Cell Culture Microplates	10

XF Media & More

Part #	Description	Quantity
100840-000	XF Calibrant	500ml bottle
102353-100	XF Base Medium	2 x 1L bottle

XF^e96 Port Layout



XF^e96 Analyzer

CONTACT SEAHORSE BIOSCIENCE

Need to order consumables?

Go to: www.seahorsebio.com/order

Need technical support?

Go to: www.seahorsebio.com/techsupport

 **Seahorse Bioscience**

XF^e96 Recommended Assay Volumes

XF MICROPLATE

Recommended assay volume: 150-275 µL per well.

When performing the medium exchange, ensure that the final volume of XF Assay Medium allows for injection volumes. The final volume in the wells during the assay must not exceed 275 µL.

XF INJECTION PORTS

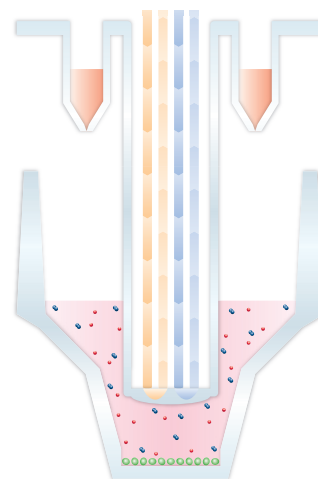
Recommended loading volume: 25 µL

(Do not exceed 30 µL due to the risk of contaminating the instrument)

Ensure that all injection ports used in the assay protocol are loaded with an equal volume to prevent failures. (i.e., if injecting compound through ports "A" and "B", all "A" and "B" ports must be loaded with either compound or assay medium).

EXAMPLE VOLUMES FOR AN XF ASSAY

Number of compound injections in assay	1	2	3	4
XF Assay Medium in wells after medium exchange	150-250 µL	150-225 µL	150-200 µL	150-175 µL
Injection Port A	25 µL	25 µL	25 µL	25 µL
Injection Port B	0	25 µL	25 µL	25 µL
Injection Port C	0	0	25 µL	25 µL
Injection Port D	0	0	0	25 µL
Total volume per well	150-275 µL			



Cutaway graphic of a single probe and well

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Seahorse Bioscience

How does a Seahorse XF Analyzer work?

Take 15 minutes to watch these six videos and you'll have a good foundation for understanding XF Assays.

Introductory videos available at <http://www.seahorsebio.com/learning/videos.php>

- a. Energy Pathways (2:06 minutes)
- b. Transient Microchamber (2:12 minutes)
- c. Drug Injection Ports (1:51 minutes)
- d. Validation 1 (2:23 minutes)
- e. Validation 2 (1:19 minutes)
- f. Measure Glycolysis (2:58 minutes)

Download software to your laptop or desktop:

<http://www.seahorsebio.com/resources/sitesearch/search.php?q=software>

XF^e96 or XF^e24 Extracellular Flux Analyzer

Wave offers a multi-touch user interface that simplifies your assay workflow. An easy 4-step process guides you through your assay and provides a succinct summary report.

Select XF^e



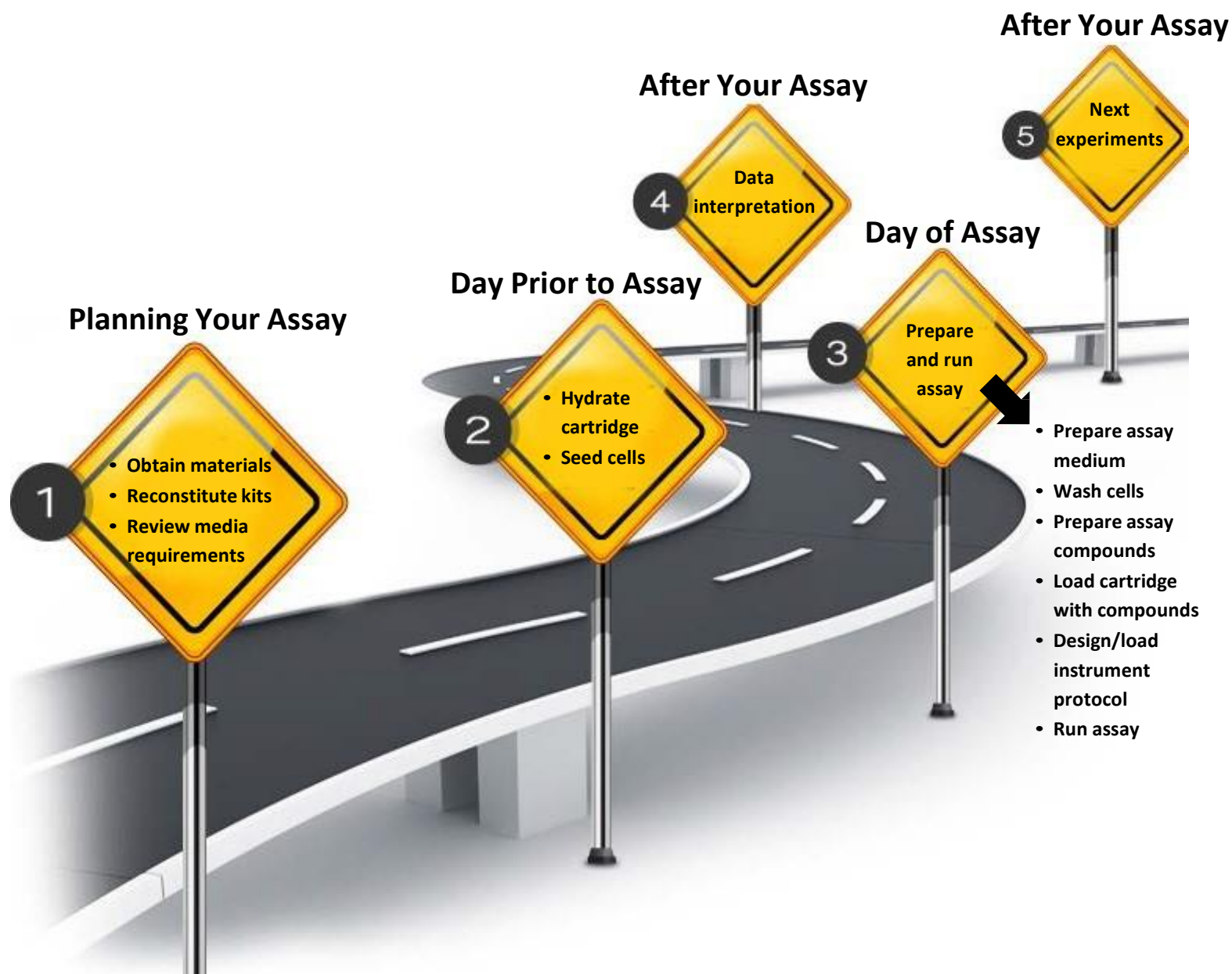
XF96, XF24 or XF24-3 Extracellular Flux Analyzer

XF software is an Excel-based system that is flexible and user-friendly. Manage experiments, acquire data, and display results to your specifications using easy-to-use XF Assay templates.

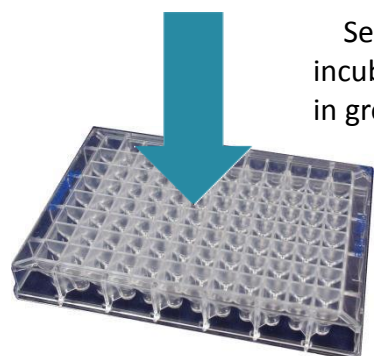
Select XF



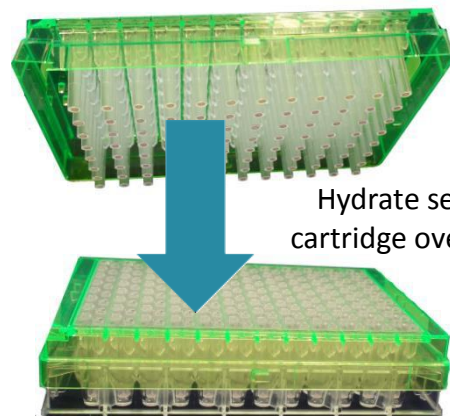
XF Assay Roadmap



Sample XF Assay Workflow Diagram



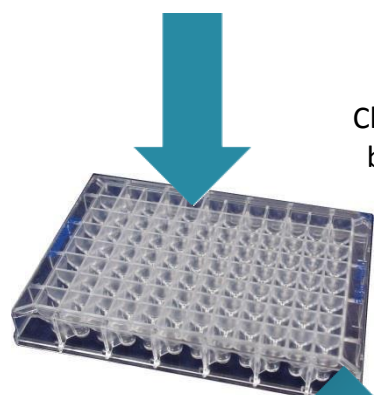
Seed cells and incubate overnight in growth medium



Hydrate sensor cartridge overnight

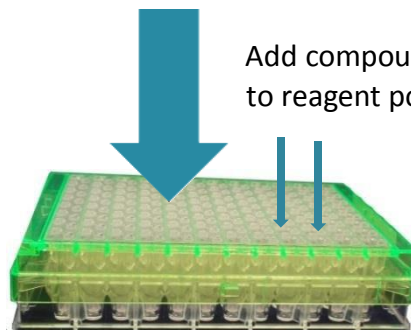
Day Before XF Assay

Day of XF Assay



Change medium to bicarbonate-free low-buffered assay medium

XF Assay



Add compounds to reagent ports

Calibrate sensors

XF^e96 Analyzer Materials List

Materials required or recommended to run a successful XF^e96 assay.

Required

The following are the materials required to run an XF^e96 assay.

- XF^e96 Analyzer with Controller
- Non-CO₂ Incubator (37°C) or XF Prep Station
- XF^e96 FluxPak (Part # 102416-001) or XF^e96 FluxPak mini (Part # 102601-100)
- pH Meter
- Inverted Phase Contrast Microscope
- P20 and P200 Pipettes and tips
- Low-buffered non-bicarbonated Medium for Assay (e.g. DMEM, KHB, or RPMI without bicarbonate)

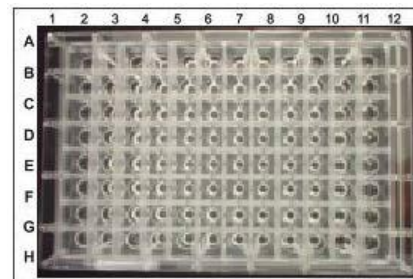
Recommended

The following are materials recommended to help you achieve optimal results. These include ensuring optimal instrument functioning, compound reconstitution, and assay experience.

- Uninterrupted power source
- Internet access
- Balance
- Stir plate
- Cell counter/Hemocytometer
- Water bath set at 37°C
- Microcentrifuge
- Touch vortex
- Centrifuge with adaptors for cell culture plates
- Reservoirs
- 15 and 50 mL conical tubes
- Sterile filter bottles (0.22 μ m filter) and cap

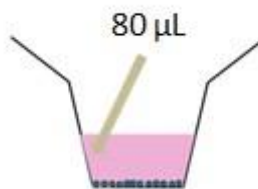
Seeding Cells in XF^e96 Cell Culture Microplates

XF assays are performed in a Seahorse 96-well XF Cell Culture Microplate in conjunction with an XF^e96 sensor cartridge. Each microplate is formatted in a typical 96-well design, as shown. The seeding surface of each well is 0.106 cm², smaller than in a typical 96-well plate, but larger than in a typical 384 well plate. This procedure describes recommendations for seeding adherent cell types for use with the XF^e96 Analyzer.



1. Harvest and re-suspend the cells to desired final concentration to seed in 80 μ L of growth medium. Optimal cell seeding numbers vary widely, though are typically between 5,000 – 40,000 cells per well and must be determined empirically.
2. Seed 80 μ L of cell suspension per well (as shown in figure below); do not seed cells in background correction wells (A1, A12, H1, H12). Be sure to put medium only (no cells) in the background correction wells.
3. Allow plate to rest at room temperature in the tissue culture hood for one hour. This will promote even cell distribution and reduce edge effects. Monitor adherence using a microscope.
4. Allow the cells to grow overnight in a cell culture incubator. Monitor growth and health of cells using a microscope.

Hint: Hold the pipette tip at an angle about halfway down the side of the wells for best technique and most homogeneous cell layer.



How to Hydrate a Seahorse XFe96 Sensor Cartridge

For use with Seahorse XF96 and XFe96 Analyzers

This document describes the Basic Procedure for preparing a sensor cartridge before running an XF assay.

Materials

Seahorse XFe96 FluxPaks containing:

- Seahorse XFe96 Extracellular Flux Assay Kit:
 - Cartridge Lid
 - Sensor Cartridge
 - Utility Plate
- Seahorse XF96 Cell Culture Microplates
- Seahorse XF Calibrant (500 mL)

Also required, but not included:

- 200 μ L pipettor and tips

Procedure

Day Prior to Assay

1. Open the Seahorse XFe96 Extracellular Flux Assay Kit and remove the contents.
2. Place the sensor cartridge upside down next to the utility plate.
3. Fill each well of the utility plate with 200 μ L of Seahorse XF Calibrant.
4. Lower the sensor cartridge onto the utility plate submerging the sensors in calibrant.
5. Verify the calibrant level is high enough to keep the sensors submerged.
6. Place in a non-CO₂ 37°C incubator overnight. To prevent evaporation of the calibrant, verify that the incubator is properly humidified.

Day of Assay

7. Following the overnight incubation, remove the cartridge assembly from the incubator.
8. Lift the sensor cartridge completely out of the calibrant and utility plate.
9. Immediately replace the sensor cartridge back onto the utility plate, submerging the sensors in calibrant. This step eliminates any bubbles that may form during the overnight hydration.

NOTICE

New steps required
for accurate calibration.

Optional Procedure for Hydrating a Seahorse XFe96 Sensor Cartridge

For use with Seahorse XFe96 and XF96 Analyzers

The Basic Procedure for hydrating a sensor cartridge may not always eliminate bubbles that may form during the overnight incubation. Bubbles can cause negative oxygen consumption rates (OCR) by interfering with the instrument calibration. If the XF data contains negative OCR data when using the Basic Procedure, we recommend the following procedure for subsequent assays:

Materials

Seahorse XFe96 FluxPaks contain:

- Seahorse XFe96 Extracellular Flux Assay Kits:
 - Cartridge Lid
 - Sensor Cartridge
 - Utility Plate
- Seahorse XF96 Cell Culture Microplates
- Seahorse XF Calibrant (500 mL)

Also required, but not included:

- 200 μ L pipettor and tips
- 50mL conical tubes
- Cell culture grade sterile water

Procedure

Day Prior to Assay

1. Aliquot at least 20mL of Seahorse XF Calibrant into a 50 mL conical tube.
2. Place this Seahorse XF Calibrant in a non-CO₂ 37°C incubator overnight.
3. Open the XFe96 Extracellular Flux Assay Kit and remove the contents.
4. Place the sensor cartridge upside down next to the utility plate.
5. Fill each well of the utility plate with 200 μ L of sterile water.
6. Lower the sensor cartridge onto the utility plate submerging the sensors in water.
7. Verify the water level is high enough to keep the sensors submerged.
8. Place assembled sensor cartridge and utility plate in a non-CO₂ 37°C incubator overnight.
9. To prevent evaporation, verify that the incubator is properly humidified

Day of Assay

1. Remove the conical tube of calibrant and assembled sensor cartridge with utility plate from incubator.
2. Place the sensor cartridge upside down next to the utility plate.
3. Remove and discard water from the utility plate.
4. Fill each well of the utility plate with 200 μ L of pre-warmed Seahorse XF Calibrant.
5. Lower the sensor cartridge onto the utility plate submerging the sensors in calibrant.
6. Place assembled sensor cartridge with utility plate in a non-CO₂ 37°C incubator for 45 – 60 minutes prior to loading drug ports of the sensor cartridge.

Preparation of Assay Media for Use in XF Assays

This basic procedure details the preparation of assay media for use with (1) XF Cell Mito Stress Test Kit, and (2) XF Glycolysis Stress Test Kit

Seahorse recommends the use non-buffered assay medium for XF assays to ensure accurate, functional measurements of metabolic phenotypes in an ambient environment. The optimal assay media for the XF Cell Mito Stress Test and the XF Glycolysis Stress Test are different. Both can be prepared starting with the XF Base Medium and adding different substrates as determined by your cell model. Substrate requirements are cell type specific and may need to be determined empirically. For the XF Cell Mito Stress Test, assay medium with user-added glucose, sodium pyruvate and glutamine is recommended (see 1). For the XF Glycolysis Stress Test, assay medium with user-added glutamine is recommended (see 2).

1. XF Cell Mito Stress Test Assay Medium

For one tissue culture plate: if rinsing manually, 100 mL of assay medium is sufficient; if rinsing using the XF Prep Station 200 mL of assay medium is sufficient.

Materials

	Source	Cat #
XF Base Medium	Seahorse Bioscience	102353-100
Glucose	Sigma	G7528
Sodium Pyruvate (powder)	Sigma	P5280
Sodium Pyruvate (liquid, 100 mM)	Sigma	S8636
L-Glutamine (200 mM)	Life Technologies	25030-081
0.2 μ M Sterile Filter		
pH Meter		
1 N NaOH		

Method

1. Warm 100 mL XF Base Medium to 37°C.
2. Add glucose for the desired final concentration using the following table.

Final glucose concentration	Grams of glucose per 100 mL
2.5 mM	0.045 g
5.5 mM	0.10 g
10 mM	0.18 g
25 mM	0.45 g

3. Add the desired amount of sodium pyruvate using powder or liquid using one of the following tables.

Addition of sodium pyruvate from powder:

Final pyruvate concentration	Grams of pyruvate per 100 mL
0.5 mM	0.0055 g
1.0 mM	0.011 g
2.0 mM	0.022 g
5.0 mM	0.055 g
10.0 mM	0.110 g

Addition of sodium pyruvate from 100 mM liquid:

Final pyruvate concentration	mL of pyruvate solution per 100 mL
0.5 mM	0.5 mL
1.0 mM	1 mL
2.0 mM	2 mL

4. Add the desired amount of L-glutamine using the following table.

Addition of L-glutamine from 200 mM liquid:

Final glutamine concentration	mL of glutamine solution per 100 mL
1 mM	0.5 mL
2 mM	1 mL
4 mM	2 mL

5. Adjust pH to 7.4 using 1 N NaOH. Note: medium will respond quickly to NaOH, use small volumes and add slowly to adjust pH.
6. Filter Sterilize with a 0.2 μ M filter.
7. Keep the XF Cell Mito Stress Test Assay Medium at 37°C until ready to use.

2. XF Glycolysis Stress Test Assay Medium

For one tissue culture plate: if rinsing manually, 100 mL of assay medium is sufficient; if rinsing using the XF Prep Station 200 mL of assay medium is sufficient.

Materials

	Source	Cat #
XF Base Medium	Seahorse Bioscience	102353-100
L-Glutamine (200 mM)	Life Technologies	25030-081
0.2 μ M Sterile Filter		
pH Meter		
1N NaOH		

Method

1. Warm 100 mL of XF Base Medium to 37°C.
2. Add the desired amount of L-glutamine according to table below.

Final glutamine concentration	mL of L-glutamine solution per 100 mL
1 mM	0.5 mL
2 mM	1 mL
4 mM	2 mL

6. Adjust the pH to 7.35 ± 0.05 using 1 N NaOH. Note: medium will respond quickly to NaOH, use small volumes and add slowly to adjust pH.
8. Filter sterilize with a 0.2 μ M filter.
9. Keep the XF Glycolysis Stress Test Assay Medium at 37°C until ready to use.

Washing Cells in XF96 Cell Culture Microplates

This procedure describes replacing the growth medium with assay medium for adherent cells grown in XF96 cell culture microplates prior to being assayed using an XF^e96 or XF96 Analyzer.

1. Warm the pre-made assay medium to 37°C. See basic procedure for Assay Media Preparation.
2. Retrieve your XF cell cartridge plate from the CO₂ incubator.
3. Look at the cells under the microscope to:
 - a. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
 - b. Ensure cells are adhered, and no gaps are present.
 - c. Make sure no cells were plated in the background correction wells.
4. Wash cells with assay medium (based on the type of XF assay you are running, use XF Cell Mito Stress Test Assay Medium or XF Glycolysis Stress Test Assay Medium)
 - a. Using a XF Prep Station
 - i. Attach bottle of assay medium to XF Prep Station. Open the Seahorse XF Prep Station software. On the “Media Change” tab, select “Do Prime”, set volume to desire final volume, for example, 180 of assay medium, and unselect “Do Rinse”.
 - ii. Place the cell plate vertically onto the tray and remove the lid.
 - iii. Press “Start”.
 - b. Without using a XF Prep Station
 - i. Remove all but 20 µL of the culture medium from each well.
 - ii. Rinse cells two times with 200 µL of assay medium.
 - iii. Add the final volume to desire final volume, for example, 160 µL of assay medium to each well for a final volume of 180 µL/well.
5. Look at cells under the microscope to ensure that cells were not washed away.
6. Place the plate in a 37°C incubator **without CO₂** for one hour prior to the assay.

Loading the XF^e96 Sensor Cartridge with Compounds

The XF^e96 Sensor Cartridge Loading Guide will help ensure consistent and accurate cartridge loading for assays on the XF^e96 Extracellular Flux Analyzers. This procedure is intended for use following cartridge hydration.

Recommended injection volume is 25 μ L. *See Recommended Volumes for XF^e96*

Requirements for Proper Compound Loading:

1. Each series of ports must contain the same volume (For example, all A ports must be filled with the same volume; all B ports must be filled with the same volume, etc.).
2. All wells, including Background Correction or blank wells, need to have vehicle or compound loaded in the port being used to ensure proper injection in all wells.
3. All compounds should be diluted with the appropriate aqueous vehicle (such as XF Base Medium) before being loaded into the sensor cartridge. *Refer to Compound Preparation guide.*
4. The hydrated XF sensor cartridge must remain in the utility plate, and be placed flat on the work surface throughout the loading procedure. Do not lift or angle the plate/cartridge away from the work surface while loading.
5. Handle the XF^e96 cartridge very carefully. Hold the base of the utility plate when transporting a cartridge. Do not hold the cartridge and utility plate between your thumb and fingers. Avoid traveling with the cartridge. To mitigate the accidental discharge of compounds prior to starting the assay, the best practice is to hydrate the cartridge and load the injection ports adjacent to the the XF^e96 Analyzer.

Loading the Sensor Cartridge with compounds:

Note: The hydrated XF assay cartridge must remain in the utility plate and be placed flat on the work surface throughout the loading procedure. Do not lift or angle the plate away from the bench while loading. Hold the base of the utility plate whenever handling the cartridge to avoid triggering discharge from the injection ports.

STEP 1

Pre-warm injection compounds to 37°C.

NOTE: It is strongly recommended that injected compounds be at pH 7.35 - 7.4 at 37°C prior to loading into the injection ports.

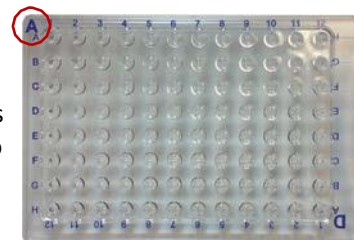
STEP 2

Orient the XF Assay Cartridge. Place row labels (lettered A-H) to the left. The triangular notch (circled in red) will be in the bottom left-hand corner.

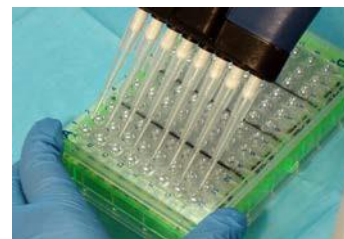


STEP 3

Place the A/D loading guide flat on top of the XF assay cartridge. Orient the loading guide so the letter 'A' (circled in red) is located in the upper left-hand corner. Use your fingertips to hold the outside edges of the loading guide to stabilize during loading so pipette tips do not dislodge the loading guide.

**STEP 4**

Using a p100 or a 10-100 μL multichannel pipette, make sure the tips are securely fitted onto the pipette. Position the pipette tips (filled with your compounds for injection) into the desired column in the loading guide, and orient the tips at a very slight angle ($<5^\circ$). Insert the tips as far as they will go without resistance into the holes and dispense the compound. Do not force the tips into the holes. *Note: See recommendations for pipettes and tips below. Automated pipettes are generally not recommended for cartridge loading, as they may lead to compound leakage through the bottom of the ports.*

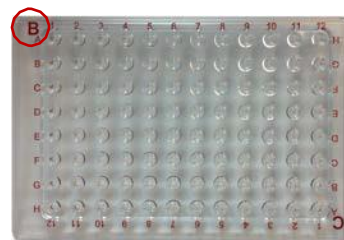
**STEP 5**

Dispense the compounds into the ports gently via a single stream. Withdraw the tips from the ports carefully, stabilizing the loading guide throughout the procedure. Avoid creating air bubbles. Do NOT tap any portion of the cartridge in an attempt to alleviate air bubbles. This may cause compound leakage from the injection port.

STEP 6

Switch to the B/C loading guide. Orient with the letter 'B' (circled in red) in the upper left-hand corner. Repeat loading procedure outlined in steps 2-4 for 'B', 'C' and 'D' injection ports, using the appropriate loading guides.

Remove and discard loading guide(s).

**STEP 7**

Position yourself at eye level with the cartridge and visually inspect the injection ports for even loading. The liquid should be down at the bottom of the port, make sure there are no residual drops on top of the cartridge. Record the position of any ports which appear uneven for later data analysis. Once all compounds have been loaded according to your experimental design, carefully transfer the cartridge (together with the utility plate) to the XF Analyzer to start calibration immediately prior to the assay.



IMPORTANT: Remove all loading guides and plate lids before inserting the cartridge into the XF Analyzer.

Recommended Pipettes and Tips:

1. Biohit Proline Plus (10-100 μL) OR BioPette Plus (20-200 μL) with:
 - a. Biohit Optifit Tip (catalog # 790351) or
 - b. VWR Ultrafine Flextop Tips (Catalog # 37001-532 (USA); 7320504 (EU)) or
 - c. Rainin 250 μL Tips (Catalog # RT-205)
2. Viaflo 300 μL (Dispense volume set to 25 μL , Dispense rate set to 8) with:
 - a. Integra 300 μL Tips (catalog # 4433)

Operation of the Prep Station for Medium Exchange

The Prep Station can be used to change the cultured medium to an assay medium before an XF experiment. After the medium exchange, the dispensed and aspiration probes are automatically washed using the DI water and cleaning fluid bottles from the back of the prep station. The tubing from the medium bottle must be manually cleaned by the user.

Materials:

Prep Station

70% ethanol (500 mL)

DI water (500 mL)

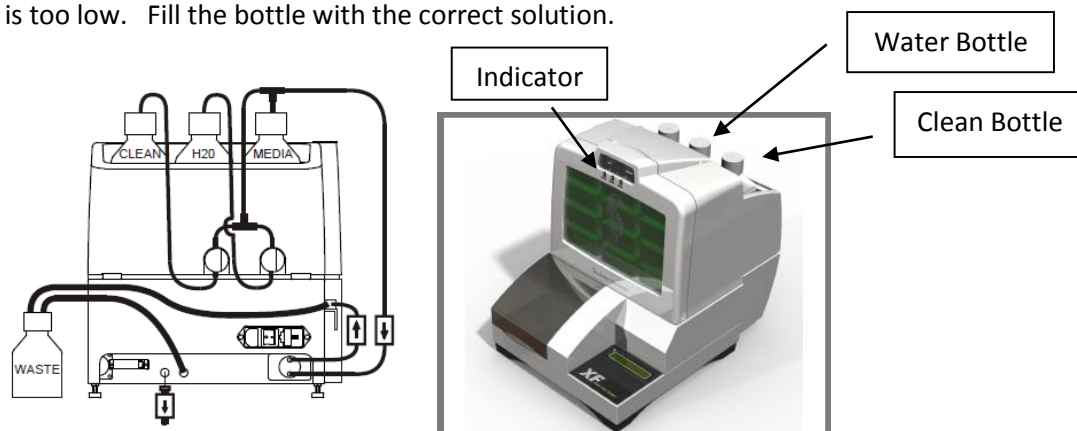
XF96 or XF24 tissue cultured plate containing adhered cells

150 mL Assay Medium (Make sure medium is warmed to 37°C in water bath)

3 – 50 mL conical tubes

Using the Prep Station to do Medium Exchange

1. Check to make sure the water bottle and clean bottle are filled with DI water and 70% ethanol respectively. If the yellow light in front of the prep station is turned on, it indicates that the volume is too low. Fill the bottle with the correct solution.



2. Check to make sure the waste bottle is not full. If it is full, empty the waste bottle according to your lab protocol.
3. Remove the empty media bottle and place the bottle containing warmed assay medium into the media slot. Remove the cap containing the tube from the empty Media bottle and place the cap containing the tube into the Assay Medium bottle.
*The medium bottle is not sterile. Make sure to remove an aliquot of the assay medium for drug dilutions.
4. Remove the lid from the tissue culture plate containing the cells.
5. Place the cells vertically (A1 facing the Seahorse logo head).

- Launch the Seahorse Prep Station software by clicking on the “Seahorse XF Prep Station” icon



- Select “Seahorse Guest.”
- Click on the “Media Change” tab
- Select “Do Prime” and type in the desired final volume in μL , for example 180 μL if using the XF96 or XFe96 Analyzer or 500 μL if using the XF24 or XFe24 Analyzer.
- Select “Do Rinse.”



- Click on “Start”

*Note: The Prep Station will prime the manifold with XF Assay Media and performs two aspirations and dispenses cycles. There will be a pause in the operation, then the prep station will perform a third aspiration and dispense cycle. Do not remove the cell plate until all 3 cycles are complete!

- After the medium exchange, remove the plate from the medium exchange station and put the lid back on top of the tissue cultured plate.
- Place the tissue cultured plate in a non- CO_2 37°C incubator or prep station until ready to use.



Non- CO_2 37°C incubator

Cleaning the Prep Station

1. Fill up two 50 mL conical tubes with DI water. Fill up one 50 mL conical tube with 70% ethanol.
2. Remove the cap containing the tube from the assay medium bottle and place the tube into one of the 50 mL conical tube containing DI water from #1.
3. Click “Prime Manifold” from the tab of the prep station software.
4. Select “Media”
5. Click “Start”



6. After the cycle is finished, transfer the tube to the 50 mL conical tube containing 70% ethanol.
7. Click “Start”
8. After the cycle is finished, transfer the tube to the second 50 mL conical tube containing DI water.
9. Click Start.
10. After the cycle is finished, put the cap containing the tube back into the empty media bottle.

Cell Density and Oligomycin Optimization with the XF Glycolysis Stress Test

This procedure describes using the XF Glycolysis Stress Test with four different cell densities and four different concentrations of oligomycin to determine the optimal cell density and oligomycin concentration to use in XF assays. In a typical cell density and oligomycin optimization assay, only three basal rate measurements followed by the oligomycin injection and three more rate measurements, are needed to determine the optimal cell seeding density and the optimal concentration of oligomycin. However, for the purposes of providing richer data for discussion, we will run the XF Glycolysis Stress Test and inject (A) Glucose, (B) Oligomycin (4 concentrations) and (C) 2-Deoxy-D-glucose (2-DG).

Plate Layout:

[Oligomycin]		0 μ M			0.5 μ M			1.0 μ M			2.0 μ M			Cell #
		1	2	3	4	5	6	7	8	9	10	11	12	
A		●	●	●	●	●	●	●	●	●	●	●	●	5 K
B		●	●	●	●	●	●	●	●	●	●	●	●	
C		●	●	●	●	●	●	●	●	●	●	●	●	10 K
D		●	●	●	●	●	●	●	●	●	●	●	●	
E		●	●	●	●	●	●	●	●	●	●	●	●	20 K
F		●	●	●	●	●	●	●	●	●	●	●	●	
G		●	●	●	●	●	●	●	●	●	●	●	●	40 K
H		●	●	●	●	●	●	●	●	●	●	●	●	

Injections:

All compounds will be made at 10x the final concentrations in the wells.

Port A: Glucose - 10 mM final concentration in the well

Port B: Oligomycin

Columns 1-3: 0 μ M final concentration in the well

Columns 4-6: 0.5 μ M final concentration in the well

Columns 7-9: 1.0 μ M final concentration in the well

Columns 10-12: 2.0 μ M final concentration in the well

Port C: 2-DG – 50 mM final concentration in the well

Protocol:

1. Warm the pre-made XF Glycolysis Stress Test Assay Medium to 37°C.
Adjust pH to 7.4 ± 0.1 at 37°C.
2. Retrieve your cell plate from the CO₂ incubator. Note the time.
3. Look at cells under the microscope to:
 - i. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
 - ii. Ensure cells are adhered, and no gaps are present.
 - iii. Make sure no cells were plated in the background correction wells.
4. Wash cells with XF Glycolysis Stress Test Assay Medium
 - i. Using a XF Prep Station
 - a. Attach bottle of XF Glycolysis Stress Test Medium to XF Prep Station. Open the Seahorse XF Prep Station software. On the “Media Change” tab, select “Do Prime”, set final volume to 180 µL of assay medium, and unselect “Do Rinse”.
 - b. Place the cell plate vertically onto the tray and remove the lid.
 - c. Press “Start”.
 - ii. Without using a XF Prep Station
 - a. Remove all but 20 µL of the culture medium from each well.
 - b. Rinse cells two times with 200 µL of assay medium.
 - c. Add assay medium to each well for a final volume of 180 µL/well.
5. Look at cells under the microscope to ensure that cells were not washed away.
6. Place the plate in a 37°C incubator **without CO₂** for one hour prior to the assay.
7. Prepare the stock compounds from the XF Glycolysis Stress Test (*For more details, refer to the XF Glycolysis Stress Test User Guide*).
 - i. **Important:** Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.
 - ii. The XF Glycolysis Stress Test Kit includes 6 foil pouches each containing oligomycin, 6 vials containing glucose and 6 vials containing 2-DG. The kit reagents are sufficient for 6 complete XF Glycolysis Stress Test assays in a 96 or 24-well XF Cell Culture Microplate.
 - iii. Remove one foil pouch containing oligomycin and 1 vial containing glucose and 1 vial containing 2-DG from the kit box.
 - iv. Allow compounds to warm to room temperature in the sealed pouch/vials for approximately 15 minutes.
 - v. Re-suspend each component with prepared assay medium in volumes described in Table 3 with a p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds. Vortex the 2-DG for at least 1 minutes to ensure that it goes into solution.

	Volume of Assay Medium	Final Concentration
Glucose	3000 µL	100 mM
Oligomycin	720 µL	100 µM
2-DG	3000 µL	500 mM

8. Prepare serial dilutions of oligomycin in assay medium, as detailed below.

Port A Oligomycin	Tube	[Final well] (μM)	Stock volume (μL)	Medium volume (μL)
	a	2.0	600	2400
	b	1.0	1500 from a	1500
	c	0.5	1000 from b	1000
	d	0	0	2000























9. Get a hydrated cartridge from the non-CO₂ incubator. Load the cartridge as outlined below.
- Port A – 10 mM Glucose final concentration in the well. **Load 20 μL** of the 10x solution into each Port A.
 - Port B – Oligomycin dilutions: **Note the layout! Load 22 μL** of the 10x solutions into each Port B according to the plan.
 - Columns 1-3: 0 μM final concentration in the well
 - Columns 4-6: 0.5 μM final concentration in the well
 - Columns 7-9: 1.0 μM final concentration in the well
 - Columns 10-12: 2.0 μM final concentration in the well
 - Port C – 50 mM 2-DG final concentration in the well. **Load 25 μL** of the 10x stock into each Port C.
10. Create or load your assay template on the XF Controller. Default Mix-Wait-Measure times are 3 min – 0 min – 3 min. Usually 3 basal rate measurements are taken prior to the first injection; then 3 rate measurements after each injection.
11. On the Run Screen, Press Start and load the cartridge.

FCCP Optimization with the XF Cell Mito Stress Test

(Note: For this assay, seed cells at the optimal cell number and use the optimal oligomycin concentration that was determined in Training Assay 1.)

The XF Cell Mito Stress Test is run with six different concentrations of FCCP to determine the optimal FCCP concentration to use in your XF assays. In a typical FCCP optimization assay, it is not necessary to inject rotenone and antimycin A. However, for the purposes of providing richer data for discussion, we will run the XF Cell Mito Stress Test and inject (A) oligomycin (optimal concentration), (B) FCCP (six different concentrations) and (C) rotenone/antimycin A.

Plate Layout:

[FCCP]	0 μM	0.125 μM	0.25 μM	0.5 μM	1.0 μM	2.0 μM						
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Injections:

All compounds will be made at 10x the final concentrations in the wells.

Port A: oligomycin: _____ μ M (optimal) final concentration in the well

Port B: FCCP

Columns 1-2: 0 μ M final concentration in the well

Columns 3-4: 0.125 μ M final concentration in the well

Columns 5-6: 0.25 μ M final concentration in the well

Columns 7-8: 0.50 μ M final concentration in the well

Columns 9-10: 1.0 μ M final concentration in the well

Columns 11-12: 2.0 μ M final concentration in the well

Port C: rotenone/antimycin A: 0.5 μ M final concentration in the well

Protocol:

1. Warm the pre-made XF Cell Mito Stress Test Assay Medium to 37°C.
Adjust pH to 7.4 ± 0.1 at 37°C.
2. Retrieve your cell plate from the CO₂ incubator. Note the time.
3. Look at cells under the microscope to:
 - a. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
 - b. Ensure cells are adhered, and no gaps are present.
 - c. Make sure no cells were plated in the background correction wells.
4. Wash cells with XF Cell Mito Stress Test Assay Medium
 - a. Using a XF Prep Station
 - i. Attach bottle of XF Cell Mito Stress Test Medium to XF Prep Station. Open the Seahorse XF Prep Station software. On the “Media Change” tab, select “Do Prime”, set final volume to 180 µL of assay medium, and unselect “Do Rinse”.
 - ii. Place the cell plate vertically onto the tray and remove the lid.
 - iii. Press “Start”.
 - b. Without using a XF Prep Station
 - i. Remove all but 20 µL of the culture medium from each well.
 - ii. Rinse cells two times with 200 µL of assay medium.
 - iii. Add assay medium to each well for a final volume of 180 µL/well.
5. Look at cells under the microscope to ensure that cells were not washed away.
6. Place the plate in a 37°C incubator **without CO₂** for one hour prior to the assay.
7. Prepare Stock Compounds
 - a. **Important:** Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.
 - b. Remove foil pouch from XF Cell Mito Stress Test Kit box. Each pouch contains reagents sufficient for a complete XF Cell Mito Stress Test in a 96 or 24 well XF Cell Culture Microplate.
 - c. Allow compounds to warm to room temp in the sealed pouch for approximately 15 minutes.
 - d. Open pouch and remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap). Place tubes in a small tube rack.
 - e. Resuspend contents of each tube with prepared assay medium in volumes described in table below with a p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds.

	Volume of Assay Medium	Final Concentration
Oligomycin	630 µL	100 µM
FCCP	720 µL	100 µM
Rotenone / AntimycinA	540 µL	50 µM

8. Prepare your compounds that you will load into the cartridge ports.

- a. Prepare 3 mL of oligomycin in assay medium to achieve the desired final concentration determined in Training experiment 1 (or use 1 μM , which works for most cell types).

Port A Oligomycin	[Final well] (μM)	Stock volume (μL)	Medium volume (μL)
	0.5	150	2,850
	1.0	300	2,700
	2.0	600	2,400

- b. Prepare serial dilutions of FCCP in assay medium, as detailed below.

Port B FCCP	Tube	[Final well] (μM)	Stock volume (μL)	Medium volume (μL)
	a	2.0	700	2,800
	b	1.0	1500 from a	1500
	c	0.5	1500 from b	1500
	d	0.25	1500 from c	1500
	e	0.125	1000 from d	1000
	f	0	0	2000

- c. Pipette 300 μL of the rotenone/antimycin A stock into a 2700 μL aliquot of assay medium.
9. Get a hydrated cartridge from the non- CO_2 incubator. Load the cartridge in each port as outlined below.
- Port A – ____ μM oligomycin final concentration in the well. **Load 20 μL** of your 10x stock into each Port A.
 - Port B – FCCP dilutions: **Note the layout! Load 22 μL** of each 10x solution into the B ports in the appropriate columns shown below.
 - Columns 1-2: 0 μM final concentration in the well
 - Columns 3-4: 0.125 μM final concentration in the well
 - Columns 5-6: 0.25 μM final concentration in the well
 - Columns 7-8: 0.50 μM final concentration in the well
 - Columns 9-10: 1.0 μM final concentration in the well
 - Columns 11-12: 2.0 μM final concentration in the well
 - Port C – 0.5 μM Rot/AA final concentration in the well. **Load 25 μL** of your 10x stock into each Port C.
10. Create or load your assay template on the XF Controller. Default Mix-Wait-Measure times are 3 min – 0 min – 3 min. Usually 3 basal rate measurements are taken prior to the first injection; then 3 rate measurements after each injection.
11. On the Run Screen, Press Start and load the cartridge.
12. When prompted by the software, replace the Utility Plate with the Cell plate. Press Continue.