Agilent Seahorse XF 3D Mito Stress Test Assay

User Guide

Agilent Seahorse XF Flex 3D Capture FluxPak-L (103864-100)

Agilent Seahorse XF Flex 3D Capture Microplate-L (103862-100)

Agilent Seahorse XF 3D Capture Screen Insert Tool (103873-100)

Agilent Seahorse XF 3D Mito Stress Test Kit (103016-100)

For use with Agilent Seahorse XF Flex analyzer only.

Notices

Document Identification

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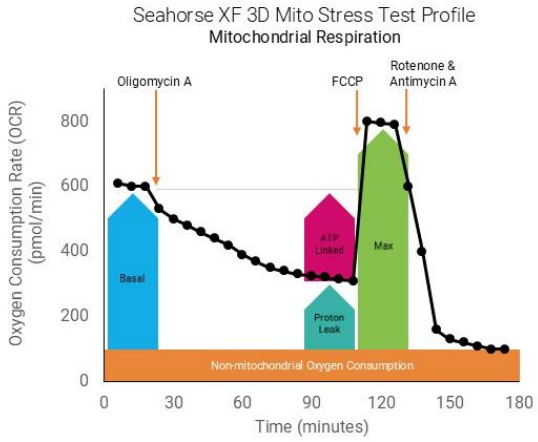
1 Introduction

Assay background

The ability to assess mitochondrial function has enabled researchers to advance their understanding of metabolism's key role in cellular physiology, disease pathology, and etiology. The Agilent Seahorse XF 3D Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of 3D samples on the Seahorse XF Flex analyzer. Built-in injection ports on Seahorse XF sensor cartridges allow for compound addition during the assay. Figure 1 illustrates the injection sequence and target action of Seahorse XF 3D Mito Stress Test modulators as well as the parameters that can be obtained. This assay provides insight into the cause of mitochondrial dysfunction within 3D models and an in-depth understanding of metabolic pathways, signals, and phenotypes. Tissue slices and other 3D samples require higher concentrations of most compounds as compared to 2D cells. To accommodate these samples, the Seahorse XF 3D Mito Stress Test kit provides higher amounts of reagents compared to the Seahorse XF Cell Mito Stress Test kit, designed for 2D cells.

A.



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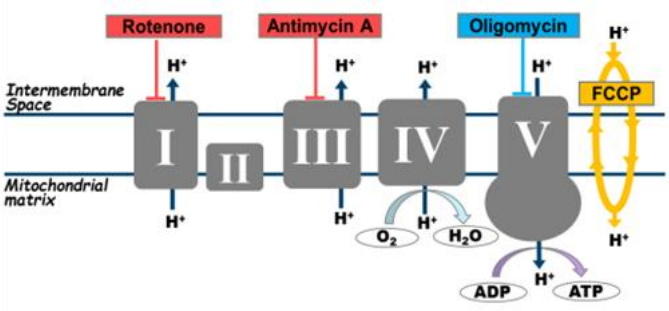


Figure 1. (A) Agilent Seahorse XF 3D Mito Stress Test profile, showing the key parameters of mitochondrial function. (B) Target action of Mito Stress Test compounds upon the electron transport chain. Oligomycin inhibits ATP synthase (complex V) blocking ATP-linked respiration. FCCP uncouples the proton gradient across the mitochondrial membrane, resulting in maximal respiration. Rotenone and antimycin A inhibit complex I and complex III, respectively, fully shutting down mitochondrial respiration.

Introduction

The Agilent Seahorse XF Flex 3D Capture Microplate-L is a customized microplate for measuring mitochondrial respiration and glycolysis in 3D samples, including various tissue samples and small model organisms. The XF Flex 3D Capture Microplate (Figure 2) is a 24-well polystyrene microplate with a 3.15 mm x 250 µm sample chamber at the bottom of each well, sized to accommodate tissue specimens or other model organisms. A specialized capture screen, optimized for proper perfusion and compound delivery, is placed on top of the sample chamber to capture the 3D sample within the microchamber allowing for repeatable, robust Seahorse XF Flex analyzer measurements.

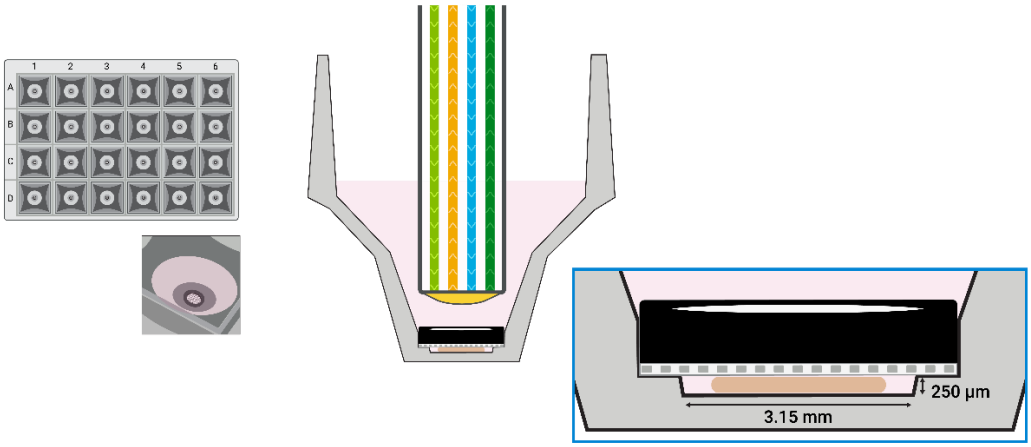


Figure 2. Agilent Seahorse XF Flex 3D Capture Microplate-L. Sample is placed within the 3.15 mm x 250 µm sample chamber and a specialized 3D capture screen is placed on top to hold samples in place.

This user guide details how to design and perform the Seahorse XF 3D Mito Stress Test using the XF Flex 3D capture microplate-L and provides initial information for data analysis.

Glossary

- Basal respiration: Oxygen consumption used to meet baseline ATP demand plus oxygen consumption resulting from mitochondrial proton leak.
- ATP-linked respiration: The decrease in oxygen consumption rate upon injection of the ATP synthase inhibitor oligomycin represents the portion of basal respiration that was being used to drive ATP production. Shows ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell.
- Proton leak: Remaining basal respiration not coupled to ATP production. Proton leak can be a sign of mitochondrial damage or can be used as a mechanism to regulate the mitochondrial ATP production.
- Maximal respiration: The maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological “energy demand” by driving the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, and amino acids) to meet this metabolic challenge. Shows the maximum rate of respiration that the cell can achieve.
- Spare respiratory capacity: This measurement indicates the capability of the cell to respond to an energetic demand beyond its baseline. The cell's ability to respond to demand can be an indicator of cell fitness or flexibility.
- Non-mitochondrial respiration: Oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after the addition of rotenone and antimycin A. Elimination of this nonspecific respiration is important to get an accurate measure of mitochondrial respiration.

2 Kit Information

Contents

The Agilent Seahorse XF 3D Mito Stress Test kit (103016-100) includes six foil pouches, each containing reagents sufficient for a full-plate assay for most 3D materials. Every pouch includes one vial each of the following compounds: oligomycin A, FCCP, and a mixture of rotenone/antimycin A. Table 1 specifies the amount of reagents in each vial.

Table 1 Agilent Seahorse XF 3D Mito Stress Test kit (103016-100) contents

Component	Cap Color	Quantity
Oligomycin A	Blue	720 nmol
FCCP	Yellow	540 nmol
Rotenone/antimycin A (Rot/AA)	Red	300 nmol each

Shipping and storage

The Agilent Seahorse XF 3D Mito Stress Test kit is shipped and stored at room temperature. The product is stable for one year from the date of manufacture. The actual expiration date is printed on the label of the assay kit box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 3 and 12 months.

Additional required items

The products in Table 2 are required for performing Seahorse XF Flex 3D assays but are not supplied with the kit.

Table 2 Additional required items

Item	Vendor	Part Number
Seahorse XF Flex Analyzer	Agilent	S7851A or S7851AN
Seahorse XF Flex 3D Capture FluxPak-L*	Agilent	103864-100
Seahorse XF DMEM assay medium pack, pH 7.4 **or Seahorse XF RPMI assay medium pack, pH 7.4** **or other media depending on tissue requirements** **(such as artificial cerebral spinal fluid: 120 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4mM KH_2PO_4, 5 mM HEPES, 10 mM glucose, pH 7.4, filter sterilized)**	Agilent	103680-100 103681-100
Seahorse XF 3D Capture Screen Insert Tool	Agilent	103873-100
Optional: Brushes for tissue handling	Precisionary Instruments	VF-VM-PB-CANAL

* The Seahorse XF Flex 3D Capture FluxPak-L contains six 24-well sensor cartridges, six Seahorse XF Flex 3D capture microplates with lids, 150 capture screens and 500 mL calibrant. All plastic components are gamma irradiated.

3 Assay Optimization Requirements

Optimal compound concentrations vary based on sample type and assay conditions. Therefore, it is recommended to perform titration experiments for the compounds provided in the kit for each new tissue or biomaterial type.

For tissue materials, tissue thickness and diameter must also be optimized. Sample preparation and handling will be dependent upon tissue type used. Optimizing sample preparation is critical to a successful Seahorse XF 3D Mito Stress Test assay. See “Frequently Asked Questions” for additional guidance.

The default Seahorse XF 3D Mito Stress Test template contains an instrument protocol with three-minute mixing, zero-minute wait and three-minute measurement time. The number of cycles is set to three cycles for basal, fifteen cycles for oligomycin A, three cycles for FCCP, and eight cycles for rotenone/antimycin A. The number of cycles required may vary for different 3D sample types, especially after oligomycin A injection. Note, assays can be monitored for compound response in real-time and the number of cycles can be changed while the assay is underway.

For assistance with any further questions, please contact Agilent Cell Analysis Technical Support.

4 Assay Workflow

One day before assay (Day 1)

Power up the Seahorse XF Flex analyzer to allow the temperature to stabilize overnight.

Prepare sensor cartridge

1. Open an Agilent Seahorse Extracellular Flux Assay kit and remove the sensor cartridge.
2. Place the sensor cartridge upside down next to the utility plate.
3. Fill each well of the utility plate with 1 mL of Seahorse XF calibrant solution and place the Seahorse XF hydrobooster on top of the utility plate. Ensure that the hydrobooster has been pressed into place.
4. Lower the sensor cartridge onto the utility plate, submerging the sensors.
5. Verify that the calibrant level is high enough to keep the sensors submerged.
6. Place the cartridge and utility plate ensemble in a 37 °C, non-CO_2 incubator overnight. To prevent evaporation of the Seahorse XF calibrant, the incubator should be humidified.

Prewarm 3D capture plate and rings

1. Place the 3D capture plate and rings in a 37 °C, non-CO_2 incubator overnight.

Design assay template

A default Seahorse XF 3D Mito Stress Test assay template is provided within the Seahorse XF Flex Controller software on the Seahorse XF Flex analyzer. You can modify the instrument protocol and experimental groups using the default template to create a custom template, if desired.

For 3D samples, modified protocols should be set to use a wait time of 00:00 and may require three to fifteen measurement cycles after compound injection.

See “Frequently Asked Questions” for additional guidance on protocol variations.

Day of assay (Day 2)

Prepare assay medium

NOTE

Assay medium is dependent upon the 3D model or tissue type used. Below are instructions for DMEM assay medium, which might not be the optimal medium for your sample.

1 Prepare the assay medium by supplementing 97 mL of Seahorse XF DMEM medium, pH 7.4, with 1.0 mL each of Seahorse XF glucose, pyruvate, and glutamine (10-, 1-, and 2-mM final concentrations in assay medium, respectively). No pH adjustment to the assay medium is necessary when standard Seahorse XF supplement concentrations are used. However, the assay medium composition can be modified if desired.

Table 3 Agilent Seahorse XF DMEM medium, pH 7.4.

Component	Volume (mL)	Final Concentration (mM)
Seahorse XF DMEM Medium, pH 7.4	97	-
Seahorse XF 1.0 M glucose solution	1	10
Seahorse XF 100 mM pyruvate solution	1	1
Seahorse XF 200 mM glutamine solution	1	2

2 Pipette 100 µL of assay medium into each well of the 3D Capture Microplate-L.

3 Warm up remaining media to 37 °C.

NOTE

BSA and serum should not be included in the media used prior to capture ring placement or in the injection ports.

Prepare the Seahorse XF Flex 3D capture plate-L with tissue samples

Sample preparation procedures vary depending on the specific tissue type used. The specimen size should be smaller than the sample chamber of the microplate (3.15 mm diameter, 250 µm height). The preparation below describes sample preparation when tissue slice punches are used.

1 After live tissue material is harvested, proceed to the next step as soon as possible or maintain tissue material in appropriate conditions to maximize viability. In some cases, this means keeping tissue in special buffers, on ice and possibly oxygenated by bubbling 95 to 100% O_2 into the maintenance medium.

2 Tissue is then prepared for slicing, using a vibrating microtome such as the Compresstome from Precisionary Instruments. This instrument creates uniform live tissue slices which can be further punched to a defined diameter, ensuring that a controlled amount of tissue is delivered to each well of the 3D capture microplate-L. Uniform tissue slices and punches provide meaningful comparisons between experimental groups and better reproducibility. Typical thickness of tissue slices is between 100–200 µm. Transfer pipets, spatulas, or fine paint brushes can be helpful tools to manipulate the tissue slices.

3 Tissue slices are then placed in cold buffer or medium, and exact punches are prepared using biopsy tools. Typical punch size is between 1–2 mm.

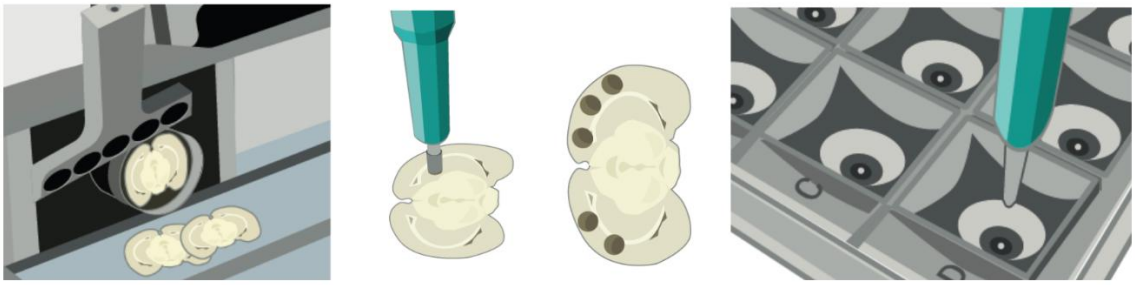


Figure 3. Example of mouse brain slice preparation, tissue punching and punch placement.

4 Tissue punches are delivered directly from the biopsy punch tool to the sample chamber at the bottom of the 3D Capture Microplate-L containing 100 µL of assay medium using the tool's plunger. Ensure tissue sample is centered in the sample chamber. Fine paint brushes may also be helpful to transfer or manipulate small tissues.

5 If planning to use imaging for data normalization, image plate prior to 3D capture screen insertion. Once screens are inserted, they may interfere with image analysis.

6 Prewet capture screens in a small petri dish for easier handling and remove any air bubbles before installation.

7 Capture screens are inserted into each well with fine tipped forceps or the Seahorse XF 3D Capture Screen Insert Tool, ensuring the mesh material is placed downward in the well towards the tissue. Press on the edges of the black ring to force the ring to sit firmly within the well. Any bubbles underneath the screen should be removed with a pipette. Visually inspect the bottom of the plate to ensure capture screens are fully inserted within all wells.

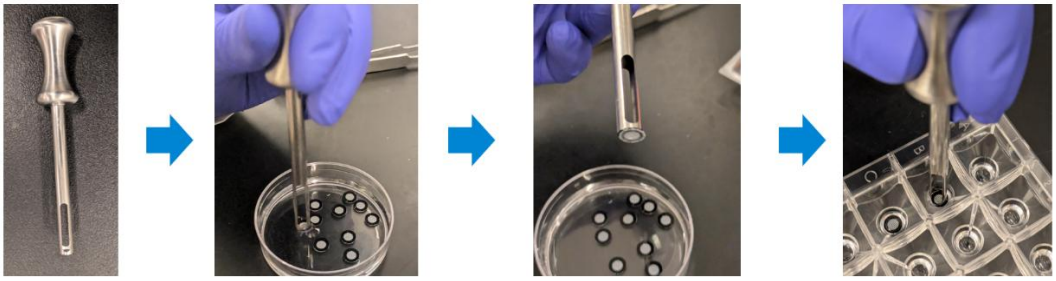


Figure 4. Capture screen insertion. Align the capture tool with a prewet capture screen and push down. Pick up the capture screen and deliver to a capture well. Push down into the bottom of the well to fully insert the capture screen.

8 Add additional assay medium to each well to bring the total volume up to 600 µL (for standard assay) or 525 µL (for assay including acute injection).

9 Place prepared plate in a 37 °C non-CO_2 incubator for 45 to 60 minutes. During this time, perform imaging (optional), prepare compounds, load sensor cartridge injection ports, and complete calibration on the Seahorse XF Flex analyzer.

Prepare compounds and load injection ports

NOTE

Use compounds on the same day that they are reconstituted. Discard any remaining compound solutions. Do not freeze and re-use

1 Prepare all compounds using the assay medium appropriate for your 3D model previously warmed at 37 °C. The assay medium used to prepare injection solutions should not contain BSA or serum.

2 Remove one foil pouch from the Seahorse XF 3D Mito Stress Test kit box. Remove the oligomycin A (blue cap), FCCP (yellow cap), and Rot/AA (red cap) vials from the pouch. Do not discard box until the part number, lot number, and software code (SW ID) have been saved.

3 Resuspend the content in each tube with assay medium in the volumes indicated in Table 4. Vortex for 30 seconds or gently pipette the solution up and down to dissolve the contents. See Frequently Asked Questions for information on modifications, including alternative protocols and stock solutions.

Table 4 Prepare stock solutions

Component	Cap Color	Assay Medium to Add (mL)	Port Injection Solution Concentration (x)	Stock Solution Concentration (µM)	Final Well Concentration (µM)
Oligomycin A	Blue	2.7	9	270	30
FCCP	Yellow	2.7	10	200	20
Rotenone/ antimycin A (Rot/AA)	Red	2.7	11	110	10

4 FCCP concentration should be optimized for specific tissue type and conditions. Use Table 5 to prepare injection solutions.

Table 5 Preparation of injection solutions. The concentration and loading volumes correspond to a starting volume of 600 µL per well in an Agilent Seahorse XF Flex 3D Capture Microplate-L.

Injection Solution	Stock Solution (µL)	Assay Medium (µL)	Port Concentration		Concentration in Well (µM)	Loading Port and Volume
			µM	Fold		
Oligomycin A	2700	0	270	9x	30	Port A: 75 µL
FCCP	750	2250	50	10x	5	Port B: 75 µL
	1500	1500	100	10x	10	
	2250	750	150	10x	15	
	2700	0	200	10x	20	
Rot/AA	2700	0	110	11x	10	Port C: 75 µL

5 Oligomycin A concentrations should also be optimized for specific tissue type and conditions. Use Table 6 to prepare injection solutions.

Table 6 Preparation of injection solutions. The concentration and loading volumes correspond to a starting volume of 600 µL per well in an Agilent Seahorse XF Flex 3D Capture Microplate-L.

	Port Concentration					
Injection Solution	**Stock Solution (µL)**	**Assay Medium (µL)**	**µM**	**Fold**	**Concentration in Well (µM)**	**Loading Port and Volume**
Oligomycin A	1000	2000	90	9x	10	Port A: 75 µL
	2000	1000	180	9x	20	
	2700	0	270	9x	30	
FCCP	Use volumes and concentrations determined to be appropriate from FCCP optimization experiment.					Port B: 75 µL
Rot/AA	2700	0	110	11x	10	Port C: 75 µL

6 To perform a modified assay including an acute injection which includes an additional injection of a test compound prior to oligomycin injection (3D Mito Stress Test (Acute)), use a starting volume of 525 µL and loading port volumes shown in Table 7.

Table 7 Preparation of injection solutions for the modified assay. The concentration and loading volumes correspond to a starting volume of 525 µL per well in an Agilent Seahorse XF Flex 3D Capture Microplate-L.

	Port Concentration	
Modified Assay	**Fold**	**Loading Port and Volume**
Test compound	8x	Port A: 75 µL
Oligomycin A	9x	Port B: 75 µL
FCCP	10x	Port C: 75 µL
Rot/AA	11x	Port D: 75 µL

7 Remove the assembled sensor cartridge with the Seahorse XF hydrobooster and utility plate from the incubator. Place the sensor cartridge upside down, next to the utility plate. Hold the utility plate steadily with one hand and use the other hand to remove the Seahorse XF hydrobooster by lifting it from one corner. Place the sensor cartridge back onto the utility plate.

8 Carefully dispense 75 µL of the oligomycin A injection solution into each port A, 75 µL of the FCCP injection solution into each port B, and 75 µL of the Rot/AA injection solution into each port C.

9 Visually inspect the injection ports for even loading. The liquid should be in the port. Make sure there are no residual drops on top of the cartridge.

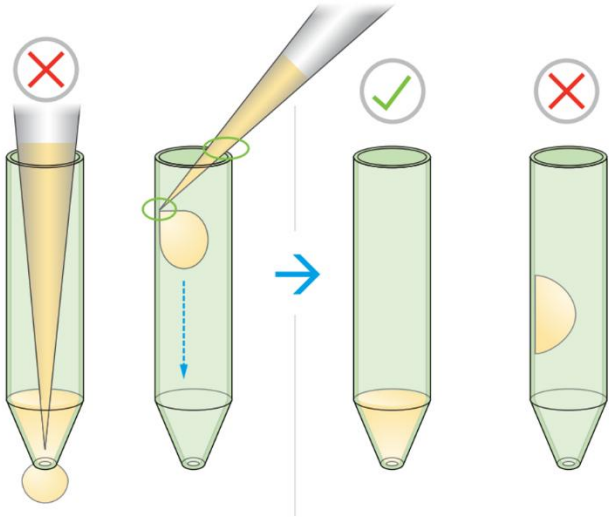


Figure 5. Injection port loading technique.

NOTE

Port A, port B, and port C for wells not intended to be used in the experiment are also required to be filled with 75 µL of assay media to ensure proper function of the instrument. Correct port loading techniques are important to ensure a successful experiment. Before any experiments, it is recommended that new users read the detailed instructions found under the "Set Up Assay > Loading Solutions" section on the Agilent Cell Analysis Learning Center website.

Running the Seahorse XF 3D Mito Stress Test

Select the 3D Mito Stress Test assay template (or 3D Mito Stress Test (Acute) template if performing an acute injection before kit reagents) available in the XF Flex controller software, and follow the instrument prompts to perform the assay

After the Seahorse XF assay is completed, save the samples to perform secondary analysis for normalization of the Seahorse XF assay data (see “Data Normalization Recommendations”).

1. Select the assay template from the list of available templates.
2. Review the groups (cell types or conditions), plate layout map, and instrument protocol for your assay; modify if desired.
3. On the review and run display, click “Scan Assay Kit” to display the assay kit information dialog.
4. If available, use the external handheld barcode wand to scan the barcode on the kit box label (outside). This will automatically add the part number and lot number of the assay kit to this dialog. Otherwise, enter manually the kit info.
5. In the SW ID field, manually type the software code on the kit box label. Click “Apply”. The software will embed this information into your data file. Click “Start Run” to start the assay.
6. When prompted, remove the cartridge lid and place the loaded sensor cartridge with the utility plate on the thermal tray. Ensure correct plate orientation and that the cartridge lid and hydrobooster have been removed. Then, click “I'm Ready”. The calibration takes approximately 15 to 20 minutes. Reminder: The Seahorse XF hydrobooster cannot be placed into the analyzer.

7 After completing calibration, click “Open Tray” to eject the utility plate and load the cell plate. Ensure that the lid is removed from the cell plate before loading.

8 Click “Load Cell Plate” to run the assay.

Data analysis

Data analysis and result reporting of the XF 3D Cell Mito Stress tests can be easily performed using Agilent XF Seahorse Analytics, a web-based software platform with dedicated assay companion views. These software features automatically calculate key parameters and present the data in convenient, customizable, and sharable graphs, simplifying your data analysis.

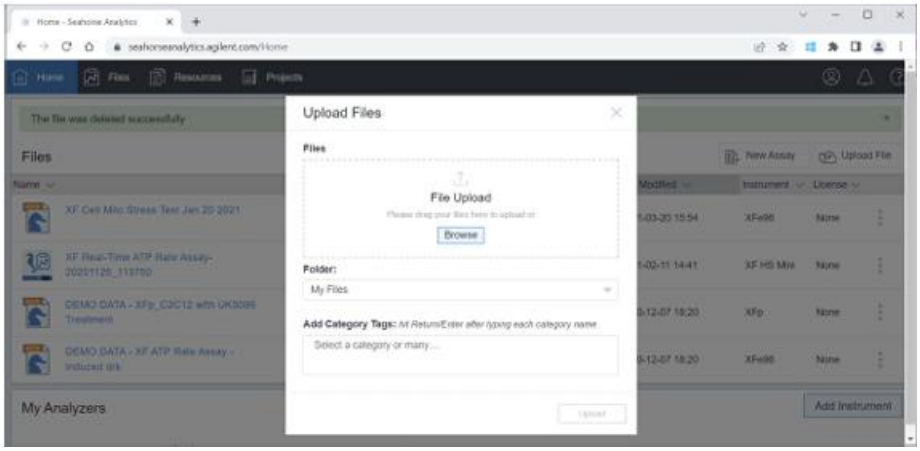
After obtaining assay result files, it is recommended to perform a data quality review using the QC tool in Wave Pro software or in Seahorse Analytics, before moving further to analyzing the data. The software will examine all the wells for outliers or unusual signals and provide a report, flagging wells with abnormal signals. Outliers, if any, can be excluded at this step.

Follow the steps below to analyze your XF 3D Mito Stress test data in Seahorse Analytics:

1 After the assay is complete, transfer your assay result file to your personal computer.

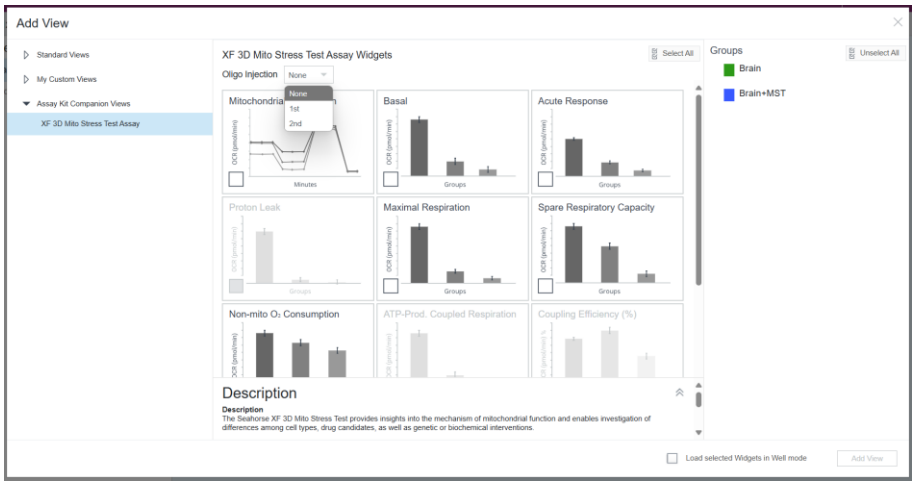
2 Register or log in to your Seahorse Analytics account at: https://seahorseanalytics.agilent.com.

3 Upload the assay result file to Seahorse Analytics. If your result file does not contain the part number, lot number, and software code information from your kit box label, manually add the kit information to the file by clicking the three-dot menu to the right of the file on the “Files” view.



4 Click the assay result file to open the “Add View” window. Under the Assay Kit Companion Views menu, select the Seahorse XF 3D Mito Stress Test analysis view.

5 The analysis tool will display specific widgets based on the design of your assay, allowing for the selection of no Oligomycin A injection (None), for a standard MST assay (1st injection) or for an acute injection assay (2nd injection). Make your selection in the Oligo injection dropdown accordingly. Select the desired analysis widgets in the View Widgets window, then click Add View at the bottom.



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6 Customized graphs are presented for review.

5 Data Normalization Options

Several methods can be used to normalize XF assay data for 3D samples. The optimal method will depend on the 3D models being studied and the experimental goal. Options for normalization of tissue punch data are outlined below. After a normalization parameter is calculated, values can be added manually using the 'Normalize' icon located in the top right corner of the Seahorse Analytics page. 

1. Total protein content per well:
   - **a** Total protein content can be evaluated using protein assays such as the Pierce BCA Assay by comparing samples to a BSA standard curve using a plate reader such as the Agilent BioTek Synergy H1. Seahorse XF data can be normalized to total or relative protein content of each well.
   - **b** If the Seahorse XF assay buffer contains BSA, remove the Seahorse XF assay buffer and wash the samples one to three times before proceeding to the next step.
   - **c** Lyse tissue in the Seahorse XF plate by adding 100 µL RIPA lysis buffer containing protease inhibitor. It is not necessary to remove the capture screen for sample processing.
   - **d** Wrap the plate in parafilm and freeze at –80 °C.
   - **e** Thaw and homogenize by vigorous pipetting through mesh. Centrifuge and transfer lysate to 96-well assay plate for BCA assay quantification of protein relative to a BSA standard curve.
2. Sample size calculated from image-based analysis:
   - **a** Prior to capture ring insertion, place the 3D capture plate with tissue slice punches in a cell imaging multimode reader such as the Agilent BioTek Cytation 1 or 5.
   - **b** Perform brightfield image acquisition and calculate area or size of the tissue punches using Gen5 imaging software. Optimization may be required based upon sample size and type.

NOTE Normalization data must be added manually. The XF Flex 3D Capture Plate-L is not compatible with the Seahorse XF Imaging and Normalization system which is intended for adherent 2D cell cultures.

6 Frequently Asked Questions

What 3D models were used to develop and validate the Seahorse XF 3D Mito Stress Test Assay with tissue samples?

Rodent brain cortex was used to develop and validate the Seahorse XF Flex 3D capture plate-L workflow. Other tissue types may require optimization in preparation and assay design.

Can the Seahorse XF Flex 3D capture plate-L be used with other 3D models?

Yes, other models may be used. These include models with the proper sizing to accommodate the geometry of the sample chamber (3.15 mm x 250 µm).

Can I change the composition of the assay media?

Yes. The media used should be tailored to the 3D model of interest. For example, some modifications that may be ideal for brain tissue include use of artificial cerebral spinal fluid as well as the inclusion of additional pyruvate alongside FCCP injection. See "A Superior System for Real-Time Metabolic Analysis with Brain Tissue and Other 3D Models" for more information. Take into consideration that optimal concentrations and drugs responses can vary depending on assay media composition.

Can I add BSA and/or serum to the assay media?

Yes. BSA and/or serum may be included in the assay media. However, BSA or serum-containing media should not be used in the injection ports. BSA should also be excluded from the 100 µL of assay media placed in the Seahorse XF Flex 3D capture plate-L prior to sample and capture ring installation. Once the capture ring is installed, additional media may contain BSA to final desired concentration. When BSA or serum is included in the Seahorse XF assay media, optimization experiments must be performed, including tissue size and compound concentration optimization. The presence of BSA or serum can often affect compound potency, and higher concentrations of compound (for example, FCCP) may be required to elicit optimal responses.

What modifications to the Seahorse XF 3D Mito Stress Test Assay template may be appropriate?

Alternative templates can be designed depending on experimental needs. For example, an acute injection of compounds (such as fuel inhibitors) may be performed before the 3D Mito Stress Test, oligomycin can be skipped for models unresponsive to the compound, or a single rotenone/antimycin A injection can be used to interrogate changes in basal respiration only. In addition, measurement time can be reduced to 2.5 minutes when high respiration rates would induce excessive oxygen depletion within a 3-minute measurement. Number of cycles can be adjusted according to kinetic responses of the injected compounds in the different tissue samples.

What injection volumes should be used for an acute injection before the 3D Mito Stress Test?

The starting volume in each well should be 525 µL and 75 µL of an 8x injection solution should be performed prior to the 3D Mito Stress Test injections. Fluid volumes less than 525 µL will compromise measurements. Ensure accurate pipetting to maintain the best data quality.

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