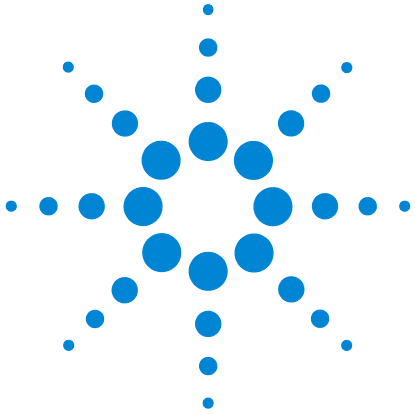

Agilent Seahorse XF Cell Mito Stress Test Kit

User Guide
Kit 103015-100

Agilent Technologies

Notices

Manual Part Number

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Contents

1 Introduction

Assay Background

The Agilent Seahorse XF Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells on the Seahorse XFe and XF Extracellular Flux Analyzers. It is a plate-based live cell assay that allows to monitor OCR in real time.

The assay uses the built-in injection ports on XF sensor cartridges to add modulators of respiration into cell well during the assay to reveal the key parameters of mitochondrial function. The modulators included in this assay kit are Oligomycin, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), Rotenone, and Antimycin. Figure 1 on page 6 illustrates the injection sequence of these modulators and the parameters can be obtained with this assay. More detailed description or definition of these parameters is provided in the "Glossary" on page 8 and the "Reference" on page 7 by Divakaruni *et al*, 2014.

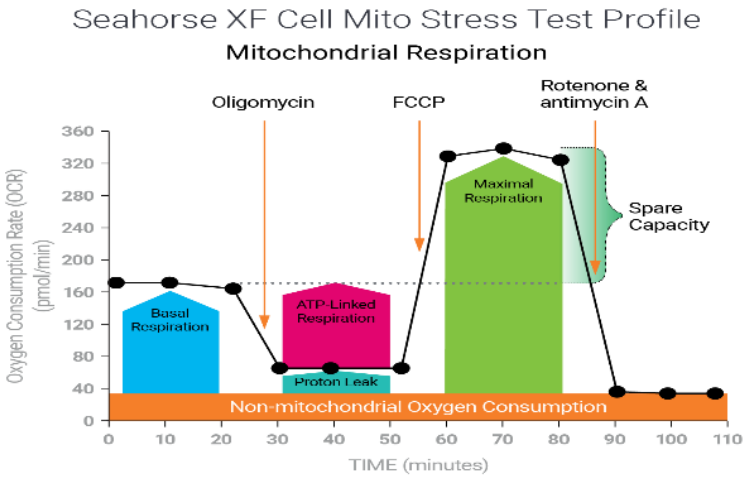

Figure 1 Agilent Seahorse XF Cell Mito Stress Test profile, showing the key parameters of mitochondrial function

Figure 2 illustrates the complexes of the Electron Transport Chain (ETC), and indicates the target of action for all the modulators included in the Seahorse XF Cell Mito Stress Test Kit.

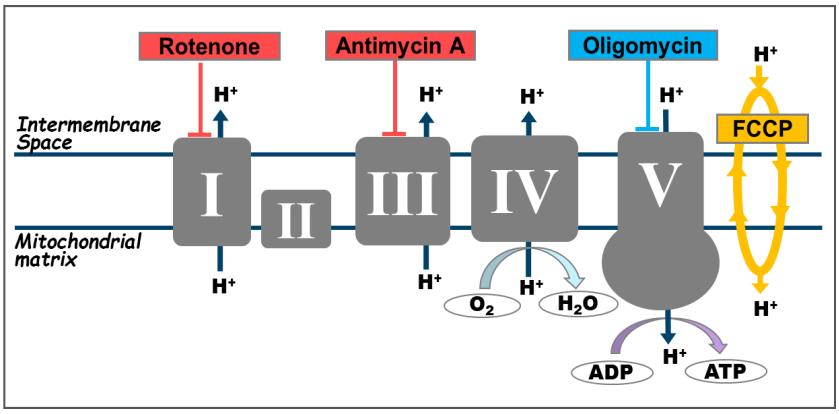

Figure 2 Agilent Seahorse XF Cell Mito Stress Test modulators of the ETC

Oligomycin inhibits ATP synthase (complex V), and is injected first in the assay following basal measurements. It impacts or decreases electron flow through the ETC, resulting a reduction in mitochondrial respiration or OCR. This decrease in OCR is linked to cellular ATP production.

Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. It is the 2nd injection following Oligomycin. As a result, electron flow through the ETC is uninhibited, and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand or under stress.

The third injection is a mixture of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This combination shuts down mitochondrial respiration and enables the calculation of nonmitochondrial respiration driven by processes outside the mitochondria.

Table 1 provides a summary of these effects.

Table 1 Summary of target and effect for the mitochondrial respiration modulators

Compound(s)	ETC target	Effect on OCR
Oligomycin	ATP synthase (complex V)	Decrease
FCCP	Inner mitochondrial membrane	Increase
Rotenone/antimycin A	Complex I and III (respectively)	Decrease

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 Seahorse XF Cell Mito Stress Test is the gold standard assay and is widely used for measuring mitochondrial function in cells. This assay provides insight into the cause of mitochondrial dysfunction and an in-depth understanding of metabolic pathways, signals, and phenotypes.

Reference

Divakaruni AS, Paradyse A, Ferrick DA, Murphy AN, Jastroch M. 2014. Analysis and Interpretation of Microplate-Based Oxygen Consumption and pH data. In Methods in Enzymology, Volume 547, Chapter 16, 309-354.

Glossary

- **Basal respiration:** Oxygen consumption used to meet cellular ATP demand resulting from mitochondrial proton leak. Shows energetic demand of the cell under baseline conditions.
- **ATP Production:** 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.
- **H+ (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 stimulating 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 as well as how closely the cell is to respiring to its theoretical maximum. The cell's ability to respond to demand can be an indicator of cell fitness or flexibility.
- **Nonmitochondrial 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. This is important to get an accurate measure of mitochondrial respiration.

2 Kit Information

Kit Contents

The Seahorse XF Cell Mito Stress Test Kit includes six foil pouches that each contain reagents sufficient for a complete Seahorse XF Cell Mito Stress Test in either the 96 or 24 well Agilent Seahorse XF Cell Culture Microplate. Every pouch includes one tube of each of the following compounds: oligomycin, FCCP, and a mix of rotenone/antimycin A. See Table 2.

Table 2 Agilent Seahorse XF Cell Mito Stress Test Kit foil pouch contents

Compound	Cap color	Quantity per tube (nmol)
Oligomycin*	Blue	63
FCCP	Yellow	72
Rotenone + antimycin A	Red	27 (of both)

* Oligomycin is a mixture of Oligomycin A, B, and C with Oligomycin A≥60%.

Kit Shipping and Storage

Product ships at ambient temperature. Product can be stored at room temperature, and is stable for one year from the date of manufacture. The expiration date is printed on the label of the kit box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 12 to 3 months.

Additional Required Items

The following items are also required for performing Seahorse XF Mito Stress Tests, but they are not supplied with the kits.

Items	Supplier	Catalog number
Agilent Seahorse XFe/XF Analyzers	Agilent Technologies	
For XFe/XF96 Analyzers: XFe96 FluxPak mini or XFe96 FluxPak	Agilent Technologies	102601-100 or 102416-100
For XFe24 Analyzers: XFe24 FluxPak mini or XFe24 FluxPak	Agilent Technologies	102342-100 or 102340-100
For XF24 Analyzers: XF24 FluxPak mini or XF24 FluxPak	Agilent Technologies	100867-100 or 100850-100
XF DMEM medium, pH 7.4* or XF RPMI medium, pH 7.4*	Agilent Technologies	103575-100 103576-100
XF 1.0 M Glucose solution	Agilent Technologies	103577-100
XF 100 mM Pyruvate solution	Agilent Technologies	103578-100
XF 200 mM Glutamine solution	Agilent Technologies	103579-100

* XF DMEM or RPMI media can also be purchased together with the supplements listed in this table as bundled products (Catalog Number 103680-100 and 103681-100). For a full list of all medium types and our recommendation for each assay kit, please refer to the Seahorse XF Media Selection Guide.
http://www.agilent.com/cs/library/selectionguide/public/5991-7878EN.pdf

Narrow p1000 pipette tips are recommended for reconstituting compounds within the tube provided (for example, Fisherbrand SureOne Micropoint Pipet Tips, p/n 02-707-402).

3 Assay Workflow

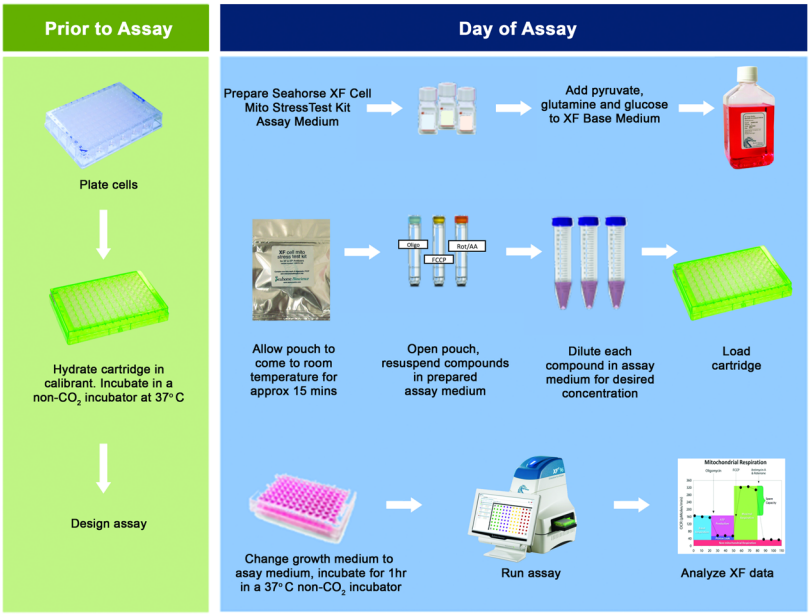

Figure 3 Agilent Seahorse XF Cell Mito Stress Test assay workflow

NOTE

Optimal cell seeding density and FCCP concentration should be empirically determined for your cell type prior to the assay. For more details, please refer to the Basic Procedures on Agilent Cell Analysis Learning Center. www.agilent.com/en/products/cell-analysis/how-to-run-an-assay

The Cell Line Reference Database is a good resource for finding information regarding the cell type or interest. www.agilent.com/cell-reference-database

Day Prior to Assay

1. Turn on the Agilent Seahorse XFe/XF Analyzer, and let it warm up overnight (minimum of five hours).
2. Plate cells at a previously determined optimized density in the Seahorse XF Cell Culture Microplate using the appropriate cell culture growth medium. For more information, refer to the Basic Procedure, “Seeding Cells in Seahorse XF Cell Culture Microplates”, available on the Agilent Cell Analysis Learning Center. www.agilent.com/en/products/cell-analysis/how-to-run-an-assay
3. Hydrate a sensor cartridge in Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight. For more information, refer to the Basic Procedure, “Hydrating the Sensor Cartridge”, on the Agilent Cell Analysis Learning Center. www.agilent.com/en/products/cell-analysis/how-to-run-an-assay
4. Design experiment in Wave. See the *Instrument User Manual* for additional details.

Day of Assay

Prepare assay medium

1. Prepare assay medium by supplementing Seahorse XF DMEM or RPMI medium. It is recommended to start with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. However, medium composition can be changed depending on cell type or the desired study conditions. For more information, refer to the Basic Procedure, “Preparing Assay medium for Use in XF Assays”, on the Agilent Cell Analysis Learning Center. www.agilent.com/en/products/cell-analysis/how-to-run-an-assay
2. Bring XF medium with pH 7.4 and XF supplements into a cell culture hood. Transfer a sufficient volume of XF medium to a sterile bottle. It is not necessary to warm the medium and supplement before this step.
3. Add proper volumes of XF supplements to achieve the desired final concentrations. This is your assay medium. When recommended supplement concentrations are used, pH-adjustment is not necessary.
4. Warm the assay medium to 37 °C in a water bath. It is ready to use.

Prepare compound stock solutions and working solutions

Important: Use the compounds on the same day that they are reconstituted. Discard any remaining compound solutions. Do not refreeze and reuse. Materials in each pouch are sufficient for a full plate assay in either 96 or 24-well format.

1. Remove one foil pouch and the decapper from Seahorse XF Cell Mito Stress Test Kit box.
2. Open pouch and remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap) with glove hand. Place the tubes in a small tube rack.
3. Remove the cap of each tube by inserting the tooth of the decapper into the inner lip of the cap, and gently rotate the tool backwards. See Figure 4 on page 14.

Figure 4 Removing reagent caps.

4 Resuspend content in each tube with prepared assay medium in volumes described in Table 3.

5 Using a pipette, gently pipette up and down the medium (~10 times) to solubilize the compounds. These are the compound stock solutions.

Table 3 Stock solutions

Compound	Volume of assay medium	Stock concentration
Oligomycin	630 µL	100 µM
FCCP	720 µL	100 µM
Rot/AA	540 µL	50 µM

6 Use the compound stock solutions to make compound working solutions for loading into the injection ports on sensor cartridges.

It is recommended to use the constant compound concentration with variable loading volume approach. This approach entails preparing the compound working solutions at a constant concentration, and requires that a different volume for each compound is loaded in the injection port.

7 Prepare 2 to 3 mL working solutions for each compound in assay medium, using the volumes indicated in Table 4 on page 15 for XFe/XF96 analyzers and in Table 5 on page 15 for XFe/XF24 analyzers.

The optimal final compound concentration for achieving maximal effect is cell line dependent, and may be affected by assay medium types. Therefore, it is recommended that, for each new cell line or assay medium, a titration experiment for the compounds is performed. This is especially important with FCCP, as the titration curve tends to be quite sharp, and too much FCCP can actually diminish responses in OCR. Follow the concentration range provided in Table 4 and Table 5 on page 15 to set up the experiment.

For Oligomycin, 1.5 µM is recommended for most cell types, while for Rot/AA, 0.5 µM is recommended. For questions, please contact Agilent Cell Analysis Technical Support.

Table 4 Compound preparation for loading to XFe/XF96 sensor cartridges. Starting assay medium volume for cell plate is 180 µL per well

	Final well (µM)	Stock solution volume (µL)	Media volume (µL)	10X (Port) (µM)	Volume added to port (µL)
Port A Oligomycin	0.5	150	2,850	5	20
	1.5	450	2,550	15	20
	2.5	630	1,890	25	20
Port B FCCP	0.125	37.5	2,962.5	1.25	22
	0.25	75	2,925	2.5	22
	0.5	150	2,850	5	22
	1.0	300	2,700	10	22
	2.0	600	2,400	20	22
Port C Rot/AA	0.5	300	2,700	5	25

Table 5 Compound preparation for loading to XFe/XF24 sensor cartridges. Starting assay medium volume for cell plate is 500 µL per well

	Final well (µM)	Stock solution volume (µL)	Media volume (µL)	10X (Port) (µM)	Volume added to port (µL)
Port A Oligomycin	0.5	150	2,850	5	56
	1.5	450	2,550	15	56
	2.5	630	1,890	25	56
Port B FCCP	0.125	37.5	2,962.5	1.25	62
	0.25	75	2,925	2.5	62
	0.5	150	2,850	5	62
	1.0	300	2,700	10	62
	2.0	600	2,400	20	62
Port C Rot/AA	0.5	300	2,700	5	69

Load solutions into the ports on sensor cartridge

Proper port-loading techniques can be found in Basic Procedure, “Loading the Sensor Cartridge with Compounds”, on the Agilent Cell Analysis Learning Center.
www.agilent.com/en/products/cell-analysis/how-to-run-an-assay

Please read the information prior to loading compounds. Ensure that the sensor cartridge is properly hydrated prior to use.

For location of the ports, please refer to Figure 5.

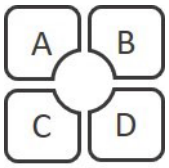

Figure 5 Location and numbering of injection ports on sensor cartridges

There are two types of assays that can be performed:

- **Standard Assay** - Only involves the injection of modulators included in the kit.
- **Modified Assay** - Includes an additional injection of a test compound prior to oligomycin injection, and Port A is used for the testing compound.

Refer to Table 6 for loading volume and port designation for compounds in different types of assays.

Table 6 Assay types, injection Port, and volume recommendation. Starting assay medium volume is 180 µL per well for 96-well cell plates, and 500 µL per well for 24-well cell plates

				Add to port volume (µL)	
Port	**Standard assay**	**Modified assay**	**Port concentration**	**96 well**	**24 well**
A	Oligomycin	Test compound*	10X	20	56
B	FCCP	Oligomycin	10X	22	62
C	Rot/AA	FCCP	10X	25	69
D	—	Rot/AA	10X	27	75

* For negative controls, assay medium should be used to replace the test compound.

Prepare Agilent Seahorse XF Cell Culture Microplate for assay

1. Remove Seahorse XF Cell Culture Microplates from 37 °C CO_2 incubator and examine the cells under a microscope to confirm confluence.
2. Remove the assay medium from water bath.
3. Change the cell culture growth medium in the cell culture microplate to warmed assay medium using a multichannel pipette, and place the cell culture microplate into a 37 °C non-CO_2 incubator for 45 minutes to 1 hour prior to the assay.

Running the Assay

Open Wave and retrieve saved assay template file. Follow the instructions below:

If you are using Wave:

1. Browse for and open the saved design file.
2. Click **Run**.
3. Place the calibration plate with the loaded sensor cartridge on the instrument tray, and click **Continue**. Calibration takes approximately 15 to 30 minutes.
   Note: Remove the cartridge lid and verify correct plate orientation.
4. When prompted, replace the calibration plate with the cell culture microplate then click **Start**.

If you are using Wave:

1. Browse for and open the saved design file, select the **Review and Run** tab, and then click **Start Run**.
2. When prompted, place the loaded sensor cartridge with the calibrant plate into the instrument, then click **I'm ready**. Calibration takes approximately 15 to 30 minutes.
   Note: Remove the cartridge lid and verify correct plate orientation.
3. Following calibration and equilibration of the cell culture microplate, when prompted click **I'm ready**.
4. Load the cell culture microplate, and click **I'm ready** to run the assay.

Data Analysis

The Seahorse XF Mito Stress Test Report Generator automatically calculates the Seahorse XF Cell Mito Stress Test parameters from Wave data that has been exported to Excel. The Seahorse XF Stress Test Report Generator can be used with either a standard or modified stress test protocol, and provides a convenient, customizable, one-page assay summary.

The Seahorse XF Report Generator can be installed either alongside Wave or directly from the Agilent Cell Analysis website. Visit https://www.agilent.com/en/products/cell-analysis/cell-analysis-software/data-analysis/seahorse-xf-cell-mito-stress-test-report-generators to learn more about the Seahorse XF Stress Test Report Generators and download the User Guide.

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