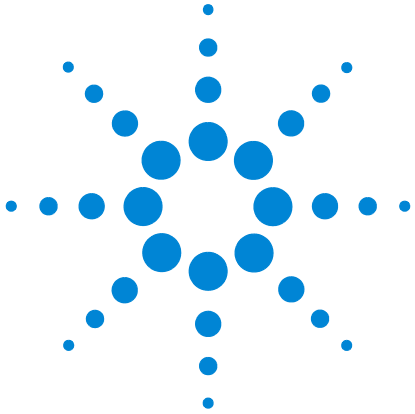

Agilent Seahorse XFp Cell Mito Stress Test Kit

User Guide
Kit 103010-100

Agilent Technologies

Notices

Manual Part Number

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Contents

1 Introduction

Assay Background

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

The assay uses the built-in injection ports on XFp sensor cartridges to add modulators of respiration into cell wells 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 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.

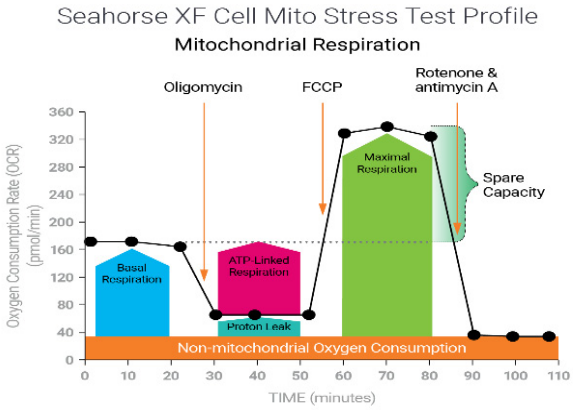

Figure 1 Seahorse XFp Cell Mito Stress Test profile, showing the key parameters of mitochondrial function.

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 XFp Cell Mito Stress Test Kit.

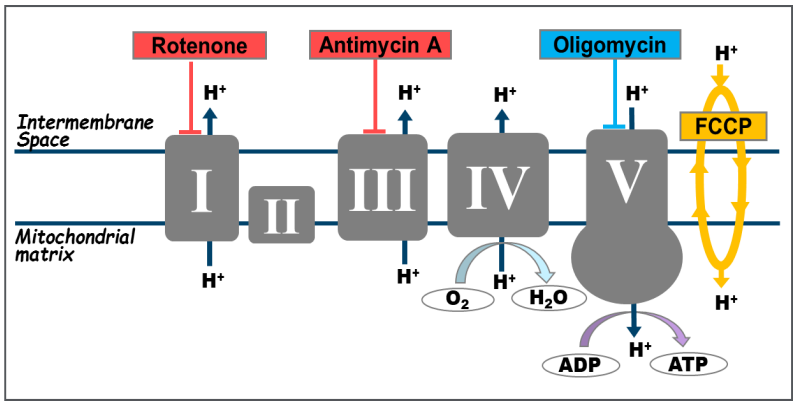

Figure 2 Agilent Seahorse XFp Cell Mito Stress Test modulators of the ETC

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 XFp 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 XFp Cell Mito Stress Test Kit includes six foil pouches that each contain reagents sufficient for a complete Seahorse XFp Cell Mito Stress Test in the Agilent Seahorse XFp Cell Culture Miniplate.

Every pouch includes one tube of each of the following compounds: oligomycin, FCCP and a mix of rotenone/antimycin A as indicated in Table 2.

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

Compound	Cap color	Quantity per tube (nmol)
Oligomycin	Blue	12.6
FCCP	Yellow	14.4
Rotenone + antimycin A	Red	5.4 (of both)

Kit Shipping and Storage

The product ships at ambient temperature. The 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 XFp Mito Stress Tests, but they are not supplied with the kits.

Table 3 Additional required items

Items	Supplier	Catalog number
Agilent Seahorse XFp Analyzers	Agilent Technologies	
Seahorse XFp FluxPak (cartridges, miniplates, and calibrant)	Agilent Technologies	103022-100
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

3 Assay

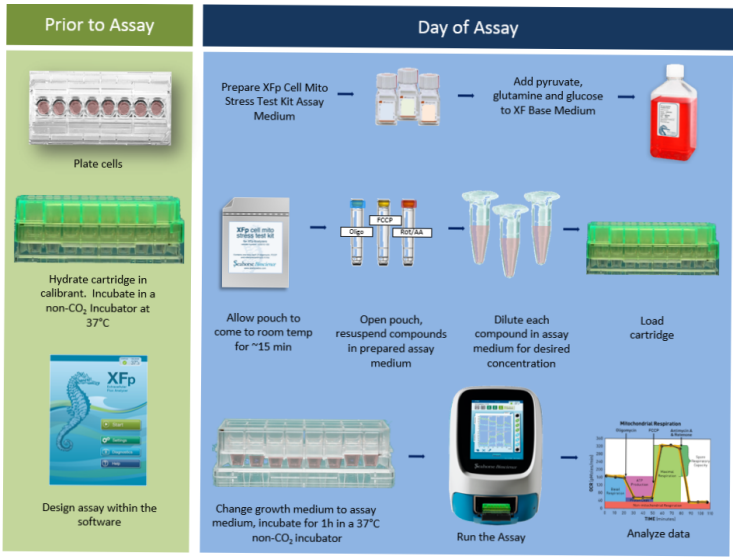

Figure 3 Agilent Seahorse XFp Cell Mito Stress Test Assay Workflow

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 XFp Analyzer, and let it warm up overnight (minimum five hours).
2. Plate the cells at a previously determined optimized density in the Seahorse XFp Cell Culture Miniplate using the appropriate cell culture growth medium.
3. Hydrate a sensor cartridge in Agilent Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight.

Day of the 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 one miniplate assay.

1 Remove one foil pouch and the decapper from Seahorse XFp Cell Mito Stress Test Kit box.

2 Open the 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.

Figure 4 Removing reagent caps

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

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

Table 4 Stock solutions.

Compound	Volume of assay medium	Stock concentration
Oligomycin	280 µL	45 µM
FCCP	288 µL	50 µM
Rot/AA	216 µL	25 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 0.3 mL working solutions for each compound in assay medium, using the volumes indicated in Table 5 for XFp 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 5 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 5 Compound preparation for loading to XFp sensor cartridges. Starting assay medium volume for cell plate is 180 µL per well.

Port designation	Final well (µM)	Stock solution volume (µL)	Media volume (µL)	10X Port (µM)	Volume added to port (µL)
Port A Oligomycin	1.5	100	200	15	20
Port B FCCP	0.125	7.5	292.5	1.25	22
	0.25	15	285	2.5	22
	0.5	30	270	5	22
	1.0	60	240	10	22
	2.0	120	180	20	22
Port C Rot/AA	0.5	60	240	5	25

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.

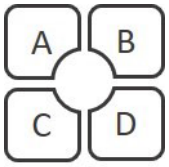

Figure 5 Location and numbering of injection ports on sensor cartridges

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 for cell plate is 180 µL per well.

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

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

Prepare Seahorse XFp Cell Culture Miniplate for assay

1. Remove cell culture miniplates from a 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 miniplate to warmed assay medium using a multichannel pipette, and place the cell culture miniplate into a 37 °C non-CO_2 incubator for 45 minutes to 1 hour prior to the assay.

Run the Assay

Run the Agilent Seahorse XFp Cell Mito Stress Test

1 Click **Start**, and select the **Agilent Seahorse Cell Mito Stress Test** default template. Click the right arrow on the Groups page, and then on the Protocol page, click **Start Assay**.

2 Place the utility plate with the loaded assay cartridge on the instrument tray, and click **Continue**. Calibration takes approximately 20 minutes.

NOTE

Remove the cartridge lid, and verify correct plate orientation.

3 When calibration has been completed, remove the utility plate and place the Seahorse XFp Cell Culture Miniplate on the tray, and press **continue** to start the assay.

NOTE

Remove the miniplate lid, and verify correct plate orientation.

Data Analysis

The Agilent Seahorse XF Cell Mito Stress Test Report Generator automatically calculates the Agilent Seahorse XFp 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 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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