

# **Agilent Seahorse XF Cell Mito Stress Test Kit**

**User Guide**  
**Kit 103015-100**

# Notices

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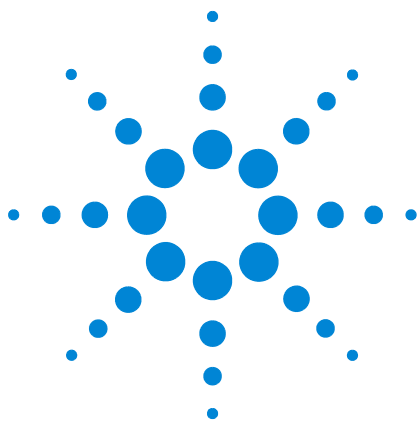
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# 1 Introduction

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## Assay Background

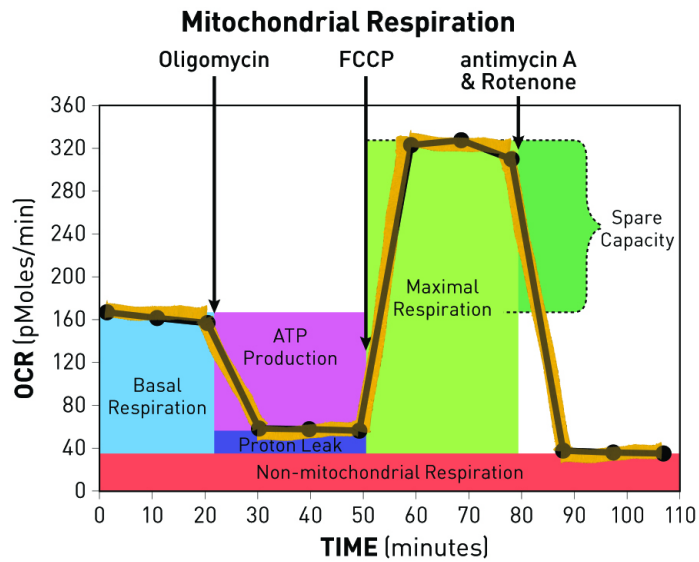
The Agilent Seahorse XF Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and nonmitochondrial respiration (see [Figure 1](#) on page 6). The Seahorse XF Cell Mito Stress Test uses modulators of respiration that target components of the electron transport chain (ETC) in the mitochondria to reveal key parameters of metabolic function. The compounds (oligomycin, FCCP, and a mix of rotenone and antimycin A) are serially injected to measure ATP production, maximal respiration, and nonmitochondrial respiration, respectively. Proton leak and spare respiratory capacity are then calculated using these parameters and basal respiration.

[Figure 2](#) on page 6 illustrates the complexes of the ETC and the target of action of all of the compounds in the Seahorse XF Cell Mito Stress Test Kit. Oligomycin inhibits ATP synthase (complex V), FCCP uncouples oxygen consumption from ATP production, and rotenone and antimycin A inhibit complexes I and III, respectively.

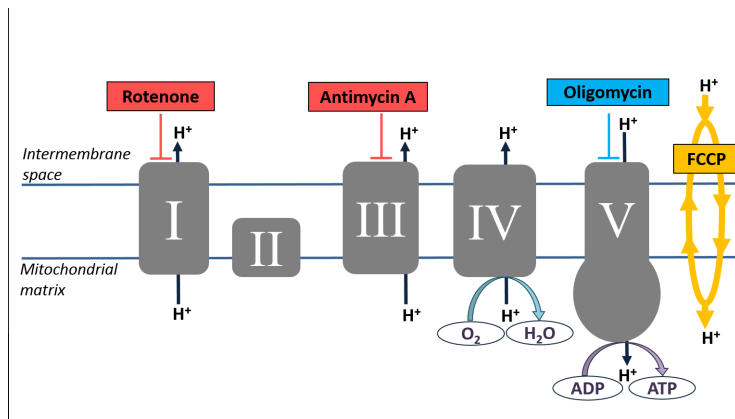
[Table 1](#) on page 7, describes how each modulator targets a specific component of the ETC. Oligomycin inhibits ATP synthase (complex V), and the decrease in OCR following injection of oligomycin correlates to the mitochondrial respiration associated with cellular ATP production. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. As a result, electron flow through the ETC is uninhibited, and oxygen is maximally consumed by complex IV.



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. The third injection is a mix 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.



**Figure 1** Agilent Seahorse XF Cell Mito Stress Test profile of the key parameters of mitochondrial respiration



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

**Table 1** Agilent Seahorse XF Cell Mito Stress Test Reagents (in order of injection)

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

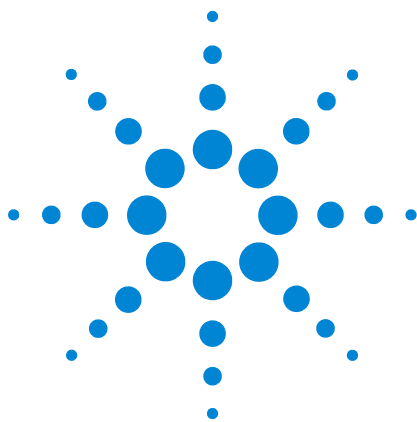
The Seahorse XF Cell Mito Stress Test is the standard assay for measuring mitochondrial function in cells. The Seahorse XF Cell Mito Stress Test Kit is designed to evaluate mitochondrial function using the Seahorse XFe and Seahorse XF Extracellular Flux Analyzers. The kit contains the reagents required to determine key parameters of mitochondrial function (See the “Glossary” on page 8 for more details.)

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. This assay provides insight into the cause of mitochondrial dysfunction and an in-depth understanding of metabolic pathways, signals, and phenotypes.

## Glossary

- **Basal respiration:** Oxygen consumption used to meet cellular ATP demand and 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<sup>+</sup> (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, 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 rotenone and antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration.





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### 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 1 year from the date of manufacture (listed on the box).



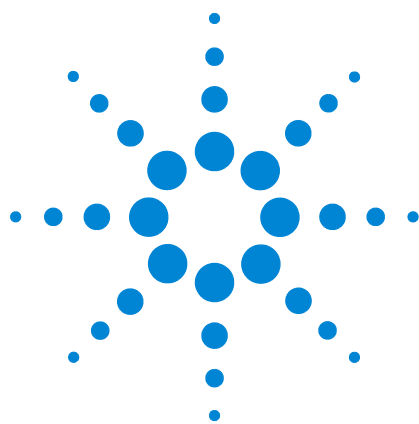
## Additional Required Items

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

Item	Supplier	Catalog Number
Agilent Seahorse XFe/XF Analyzers	Agilent Technologies	
XF Base Medium	Agilent Technologies	102353-100*
100 mM Pyruvate	Sigma	S8636 or equivalent
200 mM Glutamine	Sigma	G8540 or equivalent
2.5 M Glucose	Sigma	G8769 or equivalent

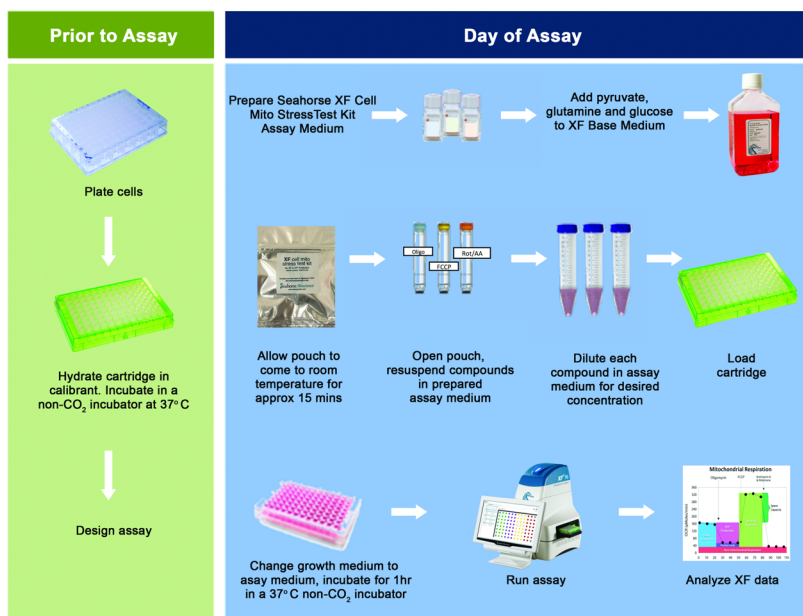
\* Please refer to the Seahorse XF Media Selection Guide for a full listing of all media types and our validated recommendations for each kit type.  
<http://www.agilent.com/cs/library/selectionguide/public/5991-7878EN.pdf>

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

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**Figure 3** Agilent Seahorse XF Cell Mito Stress Test assay workflow



**NOTE**

Optimal concentrations of FCCP and cell seeding density should be empirically determined for your cells prior to the assay. Refer to FCCP Optimization with the Seahorse XF Cell Mito Stress Test, and Seeding Cells in Seahorse XF Cell Culture Microplates Basic Procedures available on the Agilent website for more information on optimization.

The Cell Line Reference Database ([www.agilent.com/cell-reference-database](http://www.agilent.com/cell-reference-database)) is a good resource for finding reference publications for your cell type or interest.

If you are optimizing concentrations for your assay, start with the highest concentrations given in [Table 3](#) on page 15, and make serial dilutions to cover the desired concentration range.

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## 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. Refer to Basic Procedure, “Seeding Cells in Seahorse XF Cell Culture Microplates”, available on the Seahorse Bioscience website for more information.  
[www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay)
- 3 Hydrate a sensor cartridge in Seahorse XF Calibrant at 37 °C in a non-CO<sub>2</sub> incubator overnight. Refer to Basic Procedure, “Hydrating the Sensor Cartridge”, on the Seahorse website for more details.  
[www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-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 Base Medium. It is best to use 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose as a starting point; however, desired medium composition can be varied depending on cell type or *in vitro* culture conditions. Refer to Basic Procedure, “Preparing Assay Media for Use in XF Assays”, on the Seahorse Bioscience website for more information. [www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay)
- 2 Warm the assay medium to 37 °C.
- 3 Adjust the pH to 7.4 with 0.1 N NaOH. Agilent Seahorse recommends sterile filtration following pH adjustment.
- 4 Keep at 37 °C until ready to use.

### Removing reagent caps

- 1 Hold the tube in gloved hand.
- 2 Roll thumb in forward motion over the cap to loosen or, using the decapping tool provided, insert the tooth of a 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.

## Prepare stock compounds

**Important:** Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.

The Seahorse XF Cell Mito Stress Test Kit includes six foil pouches each containing three tubes of:

- Oligomycin (blue cap)
- FCCP (yellow cap)
- Rotenone/antimycin A (red cap)

Kit reagents are sufficient for six complete Seahorse XF Cell Mito Stress Test assays in the Seahorse XF cell culture microplate.

See [“Removing reagent caps”](#) on page 14 for instructions on using the decapping tool.

- 1 Remove a foil pouch, and allow the compounds to warm to room temperature in the sealed pouch for approximately 15 minutes.
- 2 Open a foil pouch, and remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap).
- 3 Place the tubes in a small tube rake.
- 4 Using a pipette, resuspend the contents of each tube with the prepared assay medium in volumes described in [Table 3](#).
- 5 Gently pipette up and down (~10 times) to solubilize the compounds.

**Table 3** Stock solution

Compound	Volume of assay medium	Final stock concentration
Oligomycin	630 $\mu$ L	100 $\mu$ M
FCCP	720 $\mu$ L	100 $\mu$ M
ROT/AA	540 $\mu$ L	50 $\mu$ M

## Prepare compounds for loading in sensor cartridge

Seahorse recommends using the constant compound concentration with variable loading volume approach. This approach entails preparing the compounds at a constant concentration and requires that a different volume of each compound be loaded in the injection port. [Table 4](#) on page 16 and [Table 5](#) on page 17 describe how to prepare to load the cartridges using this method for Seahorse XFe/XF 96 analyzers and Seahorse XFe/XF 24 analyzers, respectively.

Prepare 3 mL each compound in assay medium. Seahorse recommends using 1  $\mu$ M of oligomycin for most cells. Please contact Technical Support with any questions.

**Table 4** Compound Preparation for Loading Sensor Cartridge Ports for Seahorse XFe/XF 96 analyzers. Starting with well volume of 180  $\mu$ L assay medium

	(Final well) ( $\mu$ M)	Stock volume ( $\mu$ L)	Media volume ( $\mu$ L)	10X (Port) ( $\mu$ M)	Add to port ( $\mu$ L)
<b>Port A Oligomycin</b>	0.5	150	2,850	5	20
	1.0	300	2,700	10	20
	2.0	600	2,400	20	20
<b>Port B FCCP</b>	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
<b>Port C Rotenone/ antimycin A</b>	0.5	300	2,700	5	25



**Table 5** Compound Preparation for Loading Sensor Cartridge Ports for Seahorse XFe/XF 24 analyzers. Starting with well volume of 500  $\mu$ L assay medium

	(Final well) ( $\mu$ M)	Stock volume ( $\mu$ L)	Media volume ( $\mu$ L)	10X (Port) ( $\mu$ M)	Add to port ( $\mu$ L)
<b>Port A Oligomycin</b>	0.5	150	2,850	5	56
	1.0	300	2,700	10	56
	2.0	600	2,400	20	56
<b>Port B FCCP</b>	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
<b>Port C Rotenone/ antimycin A</b>	0.5	300	2,700	5	69

## Load sensor cartridge

Refer to Basic Procedure, “Loading the Sensor Cartridge with Compounds”, on the Seahorse Bioscience website for proper port-loading technique.

[www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay)

Load compounds into the appropriate ports of a hydrated sensor cartridge.

- **Standard Assay** - No additional injection

Port A: Oligomycin

Port B: FCCP

Port C: Rotenone/antimycin A

- **Modified Assay** - Additional injection included. To inject an additional compound prior to oligomycin, use port A for the desired compound and then load:

Port B: Oligomycin

Port C: FCCP

Port D: Rotenone/antimycin A

Table 6 lists the appropriate volumes and concentrations for this injection scheme.

**Table 6** Compound Injection Volumes Involving an Acute Injection. For Seahorse XFe/XF 96 analyzers, start with well volume of 180  $\mu$ L assay medium. For Seahorse XFe/XF 24 Analyzers, start with well volume of 500  $\mu$ L assay medium

Port	Seahorse XFe/XF 96		Seahorse XFe/XF 24	
	Add to port volume ( $\mu$ L)	Port concentration	Add to port volume ( $\mu$ L)	Port concentration
A	20	10X	56	10X
B	22	10X	62	10X
C	25	10X	69	10X
D	27	10X	75	10X

### Prepare Agilent Seahorse XF Cell Culture Microplate for assay

- 1 Remove Seahorse XF Cell Culture Microplates from 37 °C CO<sub>2</sub> 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<sub>2</sub> 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 15-30 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 15-30 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 Seahorse Bioscience website. Visit

[http://www.agilent.com/en-us/support/cell-analysis-\(seahorse\)/seahorse-xf-report-generators](http://www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-xf-report-generators) to learn more about the Seahorse XF Stress Test Report Generators and download the User Guide.

## Assay Workflow





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