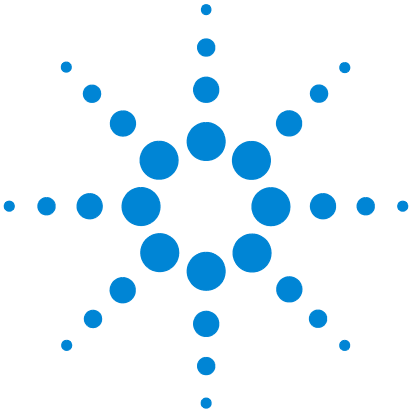

Agilent Seahorse XF Cell Energy Phenotype Test Kit

User Guide
Kit 103325-100

Agilent Technologies

Notices

Manual Part Number

103325-400

Kit Part Number

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CAUTION

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Contents

1 Introduction

Assay Background

The ability of XF technology to simultaneously measure the two major energy producing pathways of the cell - mitochondrial respiration and glycolysis - has accelerated our understanding of cellular function, activation, proliferation, differentiation, and disease etiology. The Agilent Seahorse XF Cell Energy Phenotype Test used with Agilent Seahorse XFe/XF Extracellular Flux Analyzers rapidly measures mitochondrial respiration and glycolysis under baseline and stressed conditions, to reveal the three key parameters of cell energy metabolism: Baseline Phenotype, Stressed Phenotype, and Metabolic Potential, Figure 1.

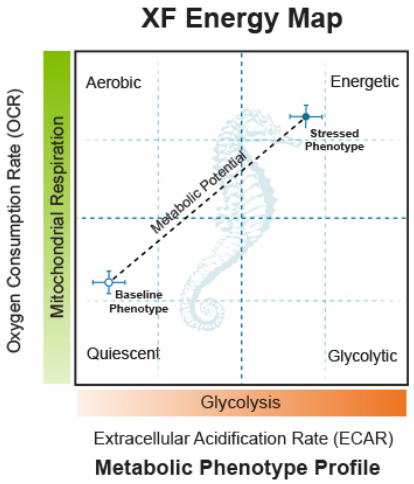

Figure 1 Agilent Seahorse XF Cell Energy Phenotype Profile
The relative utilization of the two energy pathways of a cell population is determined under both baseline (Baseline Phenotype) and stressed (Stressed Phenotype) conditions. The response to an induced energy demand is their Metabolic Potential.

The Seahorse XF Cell Energy Phenotype Test Kit provides the compounds necessary to measure the metabolic phenotypes and metabolic potential of live cells utilizing oligomycin (an inhibitor of ATP synthase) and FCCP (a mitochondrial uncoupling agent). With a simultaneous injection of these stressor compounds two events occur:

- Oligomycin inhibits ATP production by the mitochondria, and causes a compensatory increase in the rate of glycolysis as the cells attempt to meet their energy demands via the glycolytic pathway.
- FCCP depolarizes the mitochondrial membrane, and drives oxygen consumption rates higher as the mitochondria attempt to restore the mitochondrial membrane potential.

Glossary

- **Oxygen consumption rate (OCR):** The rate of decrease of oxygen concentration in the assay medium. OCR is a measure of the rate of mitochondrial respiration of the cells.
- **Extracellular acidification rate (ECAR):** The rate of increase in proton concentration (or decrease in pH) in the assay medium. ECAR is a measure of the rate of glycolysis of the cells.
- **Baseline phenotype:** OCR and ECAR of cells at starting assay conditions (specifically, in the presence of nonlimiting quantity of substrates).
- **Stressed phenotype:** OCR and ECAR of cells under an induced energy demand (specifically, in the presence of stressor compounds).
- **Metabolic potential:** Percentage increase of stressed OCR over baseline OCR, and stressed ECAR over baseline ECAR. Metabolic potential is the measure of cells' ability to meet an energy demand via respiration and glycolysis.

2 Kit Information

Kit Contents

The Agilent Seahorse XF Cell Energy Phenotype Test Kit includes 12 foil pouches, each containing reagents sufficient for a complete Seahorse XF Cell Energy Phenotype Test in one Seahorse XFe/XF Cell Culture Microplate. Table 1 shows what tubes are included in each pouch.

Table 1 Pouch contents

Compound	Cap color	Quantity per tube (nmol)
Oligomycin*	Blue	63
FCCP	Yellow	72

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

Additional Required Items

The following items are required to run the Seahorse XF Cell Energy Phenotype Tests, but not supplied with the kit.

Table 2 Additional required items

Item	Supplier	Part number
Agilent Seahorse XFe/XF Analyzer	Agilent Technologies	
Agilent Seahorse XF Base Medium	Agilent Technologies	102353-100
Agilent Seahorse XFe/XF FluxPak	Agilent Technologies	
100 mM Pyruvate	Sigma	S8636 or equivalent
200 mM Glutamine	Sigma	G8540 or equivalent
2.5 M Glucose	Sigma	G8769 or equivalent
Microfuge/Conical tubes	Various	1.5 - 15 mL capacity
Narrow p1000 pipette tips	Fisher Scientific	02-707-402 (SureOne™ Micropoint) or reagent reservoirs

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).

3 Assay Workflow

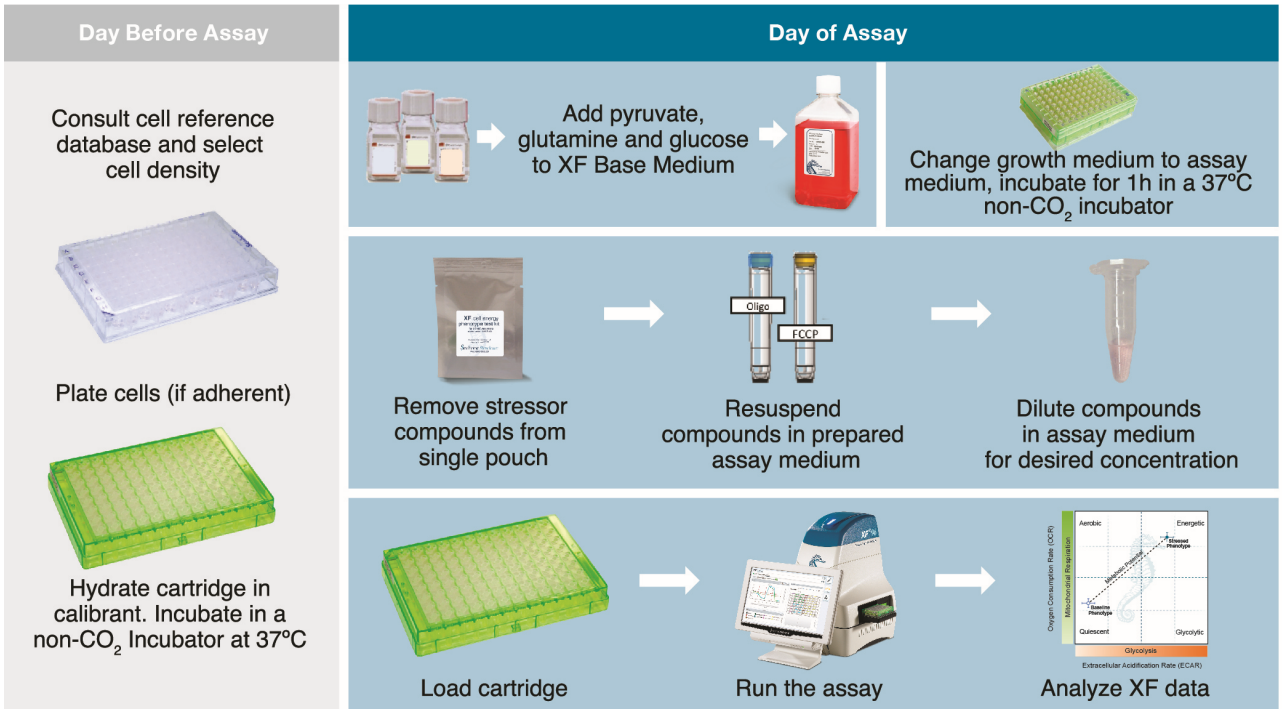

Figure 2 XF Cell Energy Phenotype Test Workflow.

Day Prior to Assay

1. Turn on the Seahorse XFe/XF Analyzer, and let it warm up overnight (minimum 5 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 *Seeding Cells in XF Cell Culture Microplates* in the Basic Procedures section, http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay
3. Hydrate a sensor cartridge in Agilent Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight. Refer to *Hydrating the Sensor Cartridge* in the Basic Procedures section, http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay
4. Download the Agilent Seahorse XF Cell Energy Phenotype Report Generator and Assay Template (for Seahorse XFe Analyzers) from the Agilent website. Select the appropriate Seahorse XFe Analyzer (Seahorse XFe96 or XFe24) when registering to download the Report Generator and accompanying Assay Template. Agilent Seahorse recommends that Seahorse XFe Analyzer users load the Assay Template provided with the Report Generator download. The default Assay Template can be modified or customized for your assay using Wave Desktop and Wave Controller. See instructions on loading the Assay Template onto your Seahorse XFe Analyzer ("Run the Agilent Seahorse XF Cell Energy Phenotype Test" on page 15). http://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Energy_Phenotype_Test_Report_Generator_User_Guide.pdf

Assay may also be designed on your Seahorse XFe Analyzer. For instructions on creating an assay using Wave Controller, refer to the Agilent Seahorse XFe Wave User Guide, http://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_B_Wave_2_3_User_Guide.pdf

Day of Assay

Prepare assay medium

1. Prepare the assay medium by supplementing Seahorse XF Base Medium. Seahorse recommends 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose as a starting point; however, the desired medium composition can be varied depending on cell type or *in vitro* culture conditions. Refer *Preparing Assay Media for Use in Seahorse XF Assays* in the Basic Procedures section, 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 (Note: Seahorse recommends sterile filtration following pH adjustment).
4. Keep at 37 °C until ready to use.

Prepare Seahorse XF Cell Culture Microplate for assay

1. Remove the Seahorse XF Cell Culture Microplates from the 37 °C CO_2 incubator, and examine the cells under a microscope to confirm confluence.
2. Remove the assay medium from water bath.
3. Remove the cell culture growth medium in the cell culture microplate, and wash with warmed assay medium using a multichannel pipette. Remove the wash, and add assay medium to inscrease the volume to 180 µL (for Seahorse XFe/XF96), or 500 µL (for Seahorse XFe/XF24). Place the cell culture microplate into a 37 °C non-CO_2 incubator for 1 hour prior to the assay.

Refer to *Washing Cells in XF Cell Culture Microplates* in the Basic Procedures section, http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay

Prepare stock compounds

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

NOTE

Refer to Figure 3 for instructions on removing the reagent caps.

1. Remove one pouch from the Seahorse XF Cell Energy Phenotype Test Kit box, and remove both tubes containing individual stressor compounds. Each pouch contains sufficient reagents for one complete Seahorse XF Cell Energy Phenotype Test in a Seahorse XF Cell Culture Microplate.
2. Using a pipette, resuspend the contents of each tube with prepared assay medium in volumes described in Table 3. Place a cap on the tube, and vortex for 1 minute to solubilize the compounds.

Table 3 Stock solutions

Compound	Quantity per tube	Volume of assay medium	Final concentration
Oligomycin	63 nmol	630 µL	100 µM
FCCP	72 nmol	720 µL	100 µM

Figure 3 Removing reagent caps
Hold the tube in gloved hand, and roll thumb in forward motion over cap to loosen or, using the decapping tool provided, insert the tooth of the decapper into the inner lip of cap, and gently rotate the tool backwards.

Combine compounds to create stressor mix

In a single tube, combine oligomycin with the appropriate amount of FCCP required by your cells to create one 10x solution using the volumes specified in Table 4.

NOTE

To verify the appropriate FCCP concentration for your cell type perform a titration by following the instructions in the Basic Procedures documentation: http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay

In most cases, oligomycin should be used at a well concentration of 1 µM.

Table 4 Stressor mix recipe

Desired FCCP concentration in well (µM)	Medium volume (µL)	Oligomycin stock volume (µL)	FCCP Stock volume (µL)	Port concentration oligomycin (µM)	Port concentration FCCP (µM)
0.125	2662.5	300	37.5	10	1.25
0.25	2625	300	75	10	2.5
0.5	2550	300	150	10	5.0
1	2400	300	300	10	10
2	2100	300	600	10	20

Load sensor cartridge

Ensure that you load the indicated volume of stressor mix into every port A of hydrated sensor cartridge.

Table 5 Starting well and compound injection volumes.

XF analyzer	Starting well volume (µL)	Add to port A (µL)
XFe/XF96	180	20
XFe/XF24	500	56

Running the Assay

Load template onto the Agilent Seahorse XFe Analyzer

(If template(s) already present, skip this step.)

Personal computer (internet access required)

1 Download the Seahorse XF Cell Energy Phenotype Report Generator from the Seahorse Bioscience website.

NOTE Select the appropriate Seahorse XFe Analyzer (XFe96 or XFe24) when registering to download the Report Generator and accompanying Assay Template.

2 Transfer the Assay Template to a USB drive or Network drive (if Seahorse XFe Analyzer is networked).

Seahorse XFe96/XFe24 Analyzer

1 Insert a USB drive in the front USB port, and wait ~10 seconds.

2 Press **Import** (bottom of the **New Assay** view).

3 Locate the Assay Template to import on the USB (or Network drive).

4 Press **Open** in the Windows dialogue box.

5 The imported Assay Template will now be available for selection in the list of available templates.

Run the Agilent Seahorse XF Cell Energy Phenotype Test

1. Select the **Seahorse XF Cell Energy Phenotype Test** Assay Template from the list of available templates, and click **Design** (or double-click the template).
   - **Groups/Conditions:** No action required - confirm or modify the default groups and conditions for your assay.
   - **Plate Map:** No action required - confirm or modify the Plate Map for your assay.
   - **Instrument Protocol:** No action required - confirm or modify the Instrument Protocol for additional measurement cycles during the assay.
   - **Review and Run:** Press **Start Run** when ready.
2. When prompted, place the loaded sensor cartridge with the calibrant plate into the Seahorse XFe Analyzer, then click **I'm Ready**. Calibration will take approximately 15-30 minutes.

NOTE Remove the cartridge lid, and verify correct plate orientation

3. Press **I'm Ready** after Calibration to load the cell culture microplate.
4. Press **I'm Ready** to close the tray door and begin the assay.

Data Analysis

The Seahorse XF Cell Energy Phenotype Test Report Generator is a critical component for analysis and interpretation of Seahorse XF Cell Energy Phenotype Test data. This Excel macro-based analytical tool automatically calculates and displays Baseline Phenotype, Stressed Phenotype, and Metabolic Potential from Seahorse XF Cell Energy Phenotype Test data in a convenient, customizable, one-page Summary Report.

To download the Seahorse XF Cell Energy Phenotype Test Report Generator and accompanying user guide visit: http://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Energy_Phenotype_Test_Report_Generator_User_Guide.pdf

The Seahorse XF Cell Energy Phenotype Test Report Generator automatically calculates the baseline phenotype, stressed phenotype, and metabolic potential of each group. It plots these data as an XF Cell Energy Phenotype and Metabolic Potential graph from XF assay data that has been exported to MS Excel.

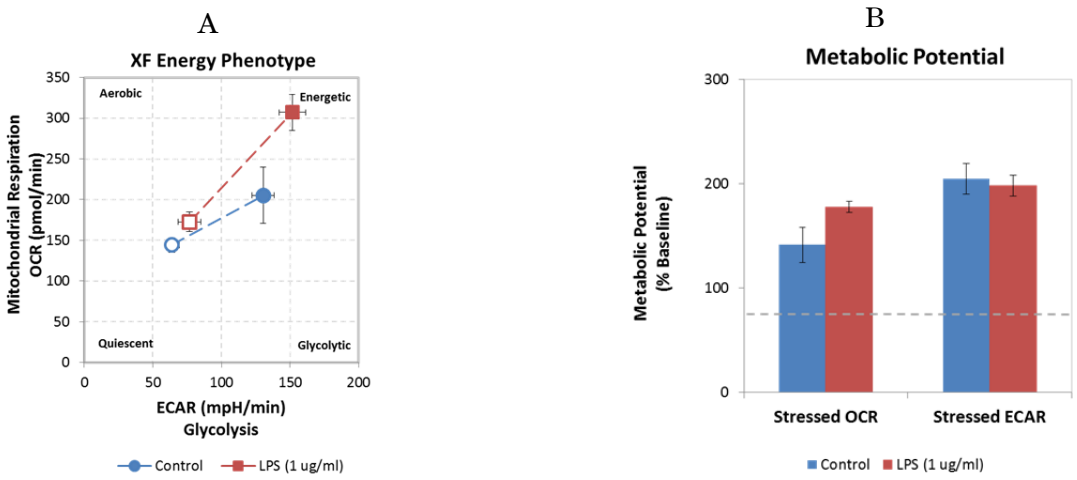

Figure 4 Macrophages become activated in response to antigens such as bacterial LPS. (A) Exposure of the RAW 264.7 macrophage cell line to LPS for 1 hour caused a small increase in baseline activity (open symbols) but a large increase in utilization of both pathways in response to mitochondrial stressors (closed symbols). (B) Priming these cells with antigen increased their aerobic potential as shown by the difference in Stressed OCR between the control (blue) and treated (red) values.

Stressed ECAR and metabolic potential

ECAR is a robust indicator of glycolysis with most cell types. However, when highly aerobic cells are stressed, CO_2 production from the mitochondria can contribute to ECAR and over-report the contribution of glycolysis to metabolic potential.

The Seahorse XF Cell Energy Phenotype Test Report Generator can help identify cells susceptible to this background CO_2 effect. Internal data from over 25 cell types has identified the **baseline OCR/ECAR ratio** as a marker of susceptibility to this effect.

When the Seahorse XF Cell Energy Phenotype Test is performed as described above (specifically, using Seahorse XF Base Medium and substrate concentrations specified):

- Cells with a baseline OCR/ECAR ratio < 4 produce CO_2 that makes a negligible contribution to ECAR, as demonstrated by empirical data from this test.
- For cells with a baseline OCR/ECAR ratio > 4, the stressed ECAR parameter could include both glycolysis and mitochondrial activity.

The Seahorse XF Glycolysis Stress Test may be run to further characterize the glycolytic capacity of the cells of interest.

Those interested in a more detailed explanation of the cellular processes contributing to ECAR are encouraged to read Section 3 of:

Divakaruni, A., Paradyse, A., Ferrick, D., Murphy, A, and Jastroch, M. "Analysis and Interpretation of Microplate-Based Oxygen Consumption and pH Data." Methods Enzymology 547 (2013): 309-354.

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