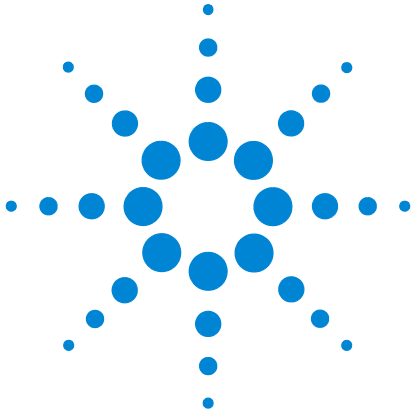

Agilent Seahorse XFp Cell Energy Phenotype Test Kit

User Guide
Kit 103275-100

Agilent Technologies

Notices

Manual Part Number

103275-400

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Contents

1 Introduction

The ability of Agilent Seahorse 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 XFp Cell Energy Phenotype Test Kit, used with the Agilent Seahorse XFp Extracellular Flux Analyzer 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.

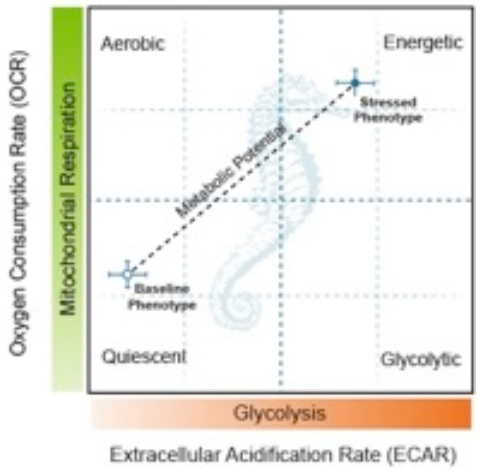

Figure 1 Agilent Seahorse XF Cell Energy Phenotype Profile
The relative utilization of the two energy pathways of a cell population is determined under both baseline (Baseline Phenotype) and stressed (Stressed Phenotype) conditions. The response to an induced energy demand is their Metabolic Potential.

Introduction

The Agilent Seahorse XFp 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 Seahorse XFp 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 XFp Cell Culture Miniplate. Table 1 shows the individual tubes each pouch contains.

Table 1 Pouch contents

Compound	Cap color	Quantity per tube (nmol)
Oligomycin*	Blue	12.6
FCCP	Yellow	14.4

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

Kit Storage

The Seahorse XFp Cell Energy Phenotype Test Kit ships at ambient temperature. It can be stored at room temperature, and is stable for 1 year from the date of manufacture (listed on the box).

Table 2 Additional required items

Item	Supplier	Part number
Agilent Seahorse XFp Analyzer	Agilent Technologies	102745-100
Agilent Seahorse XFp FluxPak (cartridges, miniplate, and calibrant)	Agilent Technologies	103022-100
Agilent Seahorse XF Base Medium	Agilent Technologies	102353-100, 103193-100
100 mM Pyruvate	Sigma	S8636 or equivalent
200 mM Glutamine	Sigma	G8540 or equivalent
2.5 M Glucose	Sigma	G8769 or equivalent

3 Assay

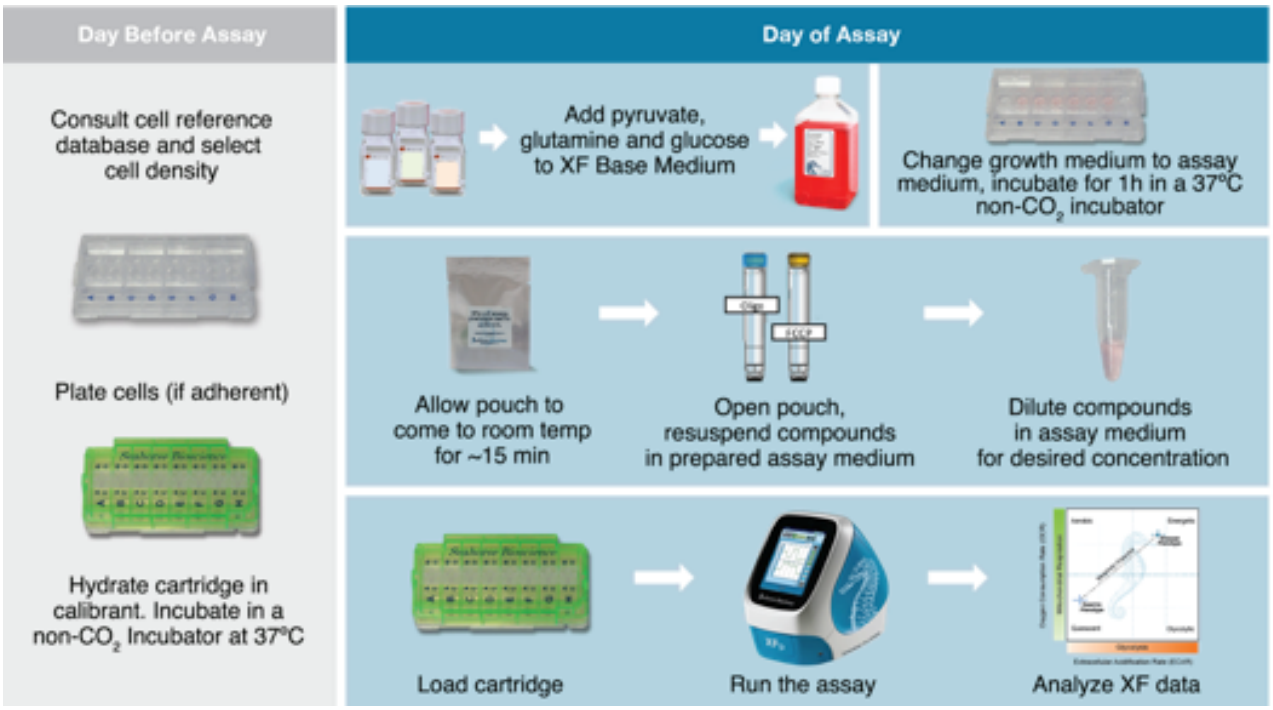

Figure 2 Agilent Seahorse XF Cell Energy Phenotype Test Workflow

Day Prior to Assay

1. Hydrate an Agilent Seahorse XFp Sensor Cartridge in Agilent Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight. (Refer to *Hydrating the Sensor Cartridge for the XFp Analyzer* in the Basic Procedures section, www.agilent.com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfp-analyzer/basic-procedures-to-run-an-xfp-assay.)
2. For adherent cells, plate the cells in the Agilent Seahorse XFp Cell Culture Miniplate at the desired density using the appropriate cell culture growth medium. Add sterile water or PBS to the moat chambers to prevent evaporation of the culture medium. (Refer to *Cell Characterization Data Table* and *Seeding Adherent Cells in XFp Cell Culture Miniplates* in the Basic Procedures section, www.agilent.com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfp-analyzer/basic-procedures-to-run-an-xfp-assay.)
3. For suspension cells, determine the desired density then plan to seed cells on the day of the assay.(Refer to *Seeding Suspension Cells in XFp Cell Culture Miniplates* in the Basic Procedures section, www.agilent.com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfp-analyzer/basic-procedures-to-run-an-xfp-assay.)

Day of Assay

Prepare assay medium

1. Prepare 20 mL assay medium by supplementing Agilent Seahorse XF Base Medium with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. Depending on your cell type these concentrations may need to be adjusted.
2. Warm the assay medium to 37 °C.
3. Adjust the pH to 7.4 with 0.1 N NaOH

NOTE Agilent Seahorse recommends sterile filtration following pH adjustment

4. Keep at 37 °C until ready to use.

Prepare Agilent Seahorse XFp Cell Culture Miniplate for assay

Adherent cells

1. Remove the Agilent Seahorse XFp Cell Culture Miniplate from the 37 °C CO_2 incubator.
2. Wash the cells with prepared assay medium by removing all but 20 µL of culture medium, and replace with assay medium.
3. Repeat step 2, then remove and replace the medium to a final volume of 180 µL per well.
4. Place the Agilent Seahorse XFp Cell Culture Miniplate into a 37 °C non-CO_2 incubator for 1 hour prior to the assay.

Suspension cells

1. Harvest, count, and resuspend the cells in assay medium at the desired density.
2. Add 50 µL assay medium to wells A and H without cells.
3. Add 50 µL cells to wells B through G.
4. Centrifuge the Agilent Seahorse XFp Cell Culture Miniplate in an XFp Carrier Tray at 300 × g for 1 minute.
5. Add 130 µL assay medium to each well, to bring the volume to 180 µL.
6. Place the Agilent Seahorse XFp Cell Culture Miniplate into a 37 °C non-CO_2 incubator for 1 hour prior to the assay.

Prepare stock compounds

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

1. Remove the foil pouch from the Agilent Seahorse XFp Cell Energy Phenotype Test Kit box. Each pouch contains reagents sufficient for a complete Agilent Seahorse XFp Cell Energy Phenotype Test in one Agilent Seahorse XFp Cell Culture Miniplate.
2. Open a pouch and remove the tubes containing oligomycin (blue cap) and FCCP (yellow cap). See Figure 3 for cap removal tip.
3. Suspend the contents of each tube with prepared assay medium in volumes described in Table 3. Pipette up and down at least 10 times to solubilize the compounds.

Figure 3 Removing reagent caps
Hold the tube in gloved hand, and roll thumb in forward motion over the 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.

Table 3 Stock solutions

	Volume of assay medium	Final concentration
Oligomycin	252 µL	50 µM
FCCP	288 µL	50 µM

Combine compounds to create stressor mix

Consult the Cell Characterization Data Table and Cell Reference Database (both available on the Agilent Technologies website: www.agilent.com), to select an appropriate FCCP concentration, based on the general class of cells you are phenotyping. Combine oligomycin and FCCP in a single tube to create a 10x solution using the volumes specified in Table 4.

NOTE

If OCR does not increase following injection, perform a FCCP titration to verify the appropriate concentration for your cell type by following the instructions in the Basic Procedure: New Cell Characterization, http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfp-analyzer/basic-procedures-to-run-an-xfp-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	232.5	60	7.5	10	1.25
0.25	225	60	15	10	2.5
0.5	210	60	30	10	5.0
1	180	60	60	10	10
2	120	60	120	10	20

Load sensor cartridge

Load 20 µL stressor mix into every port A of the hydrated sensor cartridge. Load all eight ports ‘A’.

Running the Test

Run the Agilent Seahorse XF Cell Energy Phenotype test

1 Press **Start** on the Agilent Seahorse XFp Analyzer, and select the Agilent Seahorse XF Cell Energy Phenotype Test template,* OR choose the Agilent Seahorse XF Cell Mito Stress Test assay template, and make the following modifications:

   a Deselect the FCCP and Rotenone/antimycin A injection steps.

   b Adjust the number of cycles after the oligomycin injection to 5. The protocol should look similar to the one shown in Figure 4.

Figure 4 Protocol screens.

2 If desired, adjust groups, assay results file name, and notes.

3 Click **Start Assay**.

4 Place the utility plate with the loaded sensor cartridge on the instrument tray.

NOTE

Remove the cartridge lid and verify correct orientation.

5 Click **Continue**. Calibration takes approximately 20 minutes.

6 When calibration is complete, remove the utility plate and place the prepared Agilent Seahorse XFp Cell Culture Miniplate on the tray. Note: Remove miniplate lid and verify correct plate orientation.

7 Click **Continue** to start the assay.

*If you do not have the Agilent Seahorse XF Cell Energy Phenotype Test template on your Agilent Seahorse XFp Analyzer, register and download the XF Cell Energy Phenotype Test Report Generator and the template file will be included in the downloaded folder.

Data Analysis

During the run, the injection of stressor mix typically causes both OCR and ECAR to increase, as shown in the kinetic trace in the **Overview** tab. The phenotype can be easily monitored by selecting the **OCR vs. ECAR** tab.

Figure 5 Overview and OCR vs. ECAR tabs.

Completing the test

Upon completion of the run, export the data to a USB (thumb) drive or network location. Results can be exported in Wave Assay Results format or as a MS Excel workbook.

Post-run analysis

The Agilent Seahorse XF Cell Energy Phenotype Test Report Generator, which can be downloaded from www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-xf-report-generators, 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 the XFp assay data that has been exported to MS Excel.

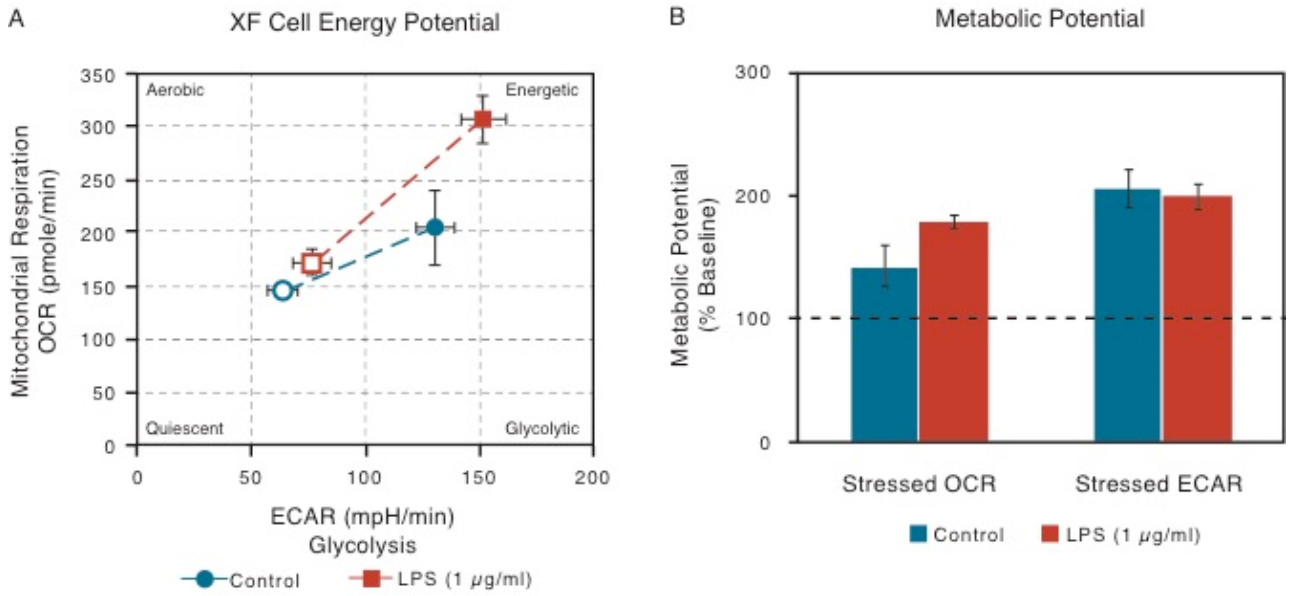

Figure 6 Macrophages become activated in response to antigens such as bacterial LPS. (A) Exposure of the RAW 264.7 macrophage cell line to LPS for 1 hour caused a small increase in baseline activity (open symbols) but a large increase in utilization of both pathways in response to mitochondrial stressors (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 Agilent 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 Agilent 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. For a more detailed explanation of the cellular processes contributing to ECAR users are encouraged to read Section 3 of the following paper.

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.

Assay

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