

Agilent Seahorse XFp Mito Fuel Flex Test Kit

**For use with Agilent Seahorse
XFp Extracellular Flux Analyzers**

**User Manual
Kit 103270-100**



Agilent Technologies

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Assay Background

The Agilent Seahorse XF Mito Fuel Flex Test is a method for measuring mitochondrial fuel usage in live cells. In combination with the Agilent Seahorse XFp Analyzer, the Agilent Seahorse XF Mito Fuel Flex Test Kit measures the dependency, capacity, and flexibility of cells to oxidize three mitochondrial fuels:

- Glucose (pyruvate)
- Glutamine (glutamate)
- Long-chain fatty acids.

The Seahorse XF Mito Fuel Flex Test determines the rate of oxidation of each fuel by measuring mitochondrial respiration [the oxygen consumption rate, OCR] of cells in the presence or absence of fuel pathway inhibitors (Figure 1 on page 6). Sequentially inhibiting the pathway of interest followed by the two alternative pathways enables the calculation of how dependent the cells are on the pathway of interest to meet basal energy demand (Figure 2 on page 7). Dependency indicates that the cells' mitochondria are unable to compensate for the blocked pathway by oxidizing other fuels. Inhibiting the two alternative pathways followed by the pathway of interest enables the calculation of cells' mitochondrial capacity to meet energy demand (Figure 3 on page 8). Fuel Flexibility is calculated by subtracting the Fuel Dependency from the Fuel Capacity for the pathway of interest (Figure 4 on page 9). Flexibility indicates the cells' mitochondria have the ability to compensate for the inhibited pathway by utilizing other pathways to fuel mitochondrial respiration. The presence of dependency and absence of flexibility demonstrates that the mitochondria require that fuel pathway to maintain basal OCR.

The Seahorse XF Mito Fuel Flex Test Kit contains three pathway inhibitors required to determine the dependency, capacity, and flexibility of cells for glucose, glutamine and long chain fatty acids.



- **UK5099** - An inhibitor of the glucose oxidation pathway. UK5099 blocks the mitochondrial pyruvate carrier (MPC). Cells convert glucose to pyruvate through glycolysis. Pyruvate can be transported into the mitochondria and oxidized by the TCA cycle.
- **BPTES** - An inhibitor of the glutamine oxidation pathway. BPTES is an allosteric inhibitor of glutaminase (GLS1). Glutaminase converts glutamine to glutamate, glutamate is then converted to alpha-ketoglutarate and oxidized by the TCA cycle.
- **Etomoxir** - An inhibitor of long chain fatty acid oxidation. Etomoxir inhibits carnitine palmitoyl-transferase 1A (CPT1A), which is critical for translocating long chain fatty acids from the cytosol into the mitochondria for beta oxidation.

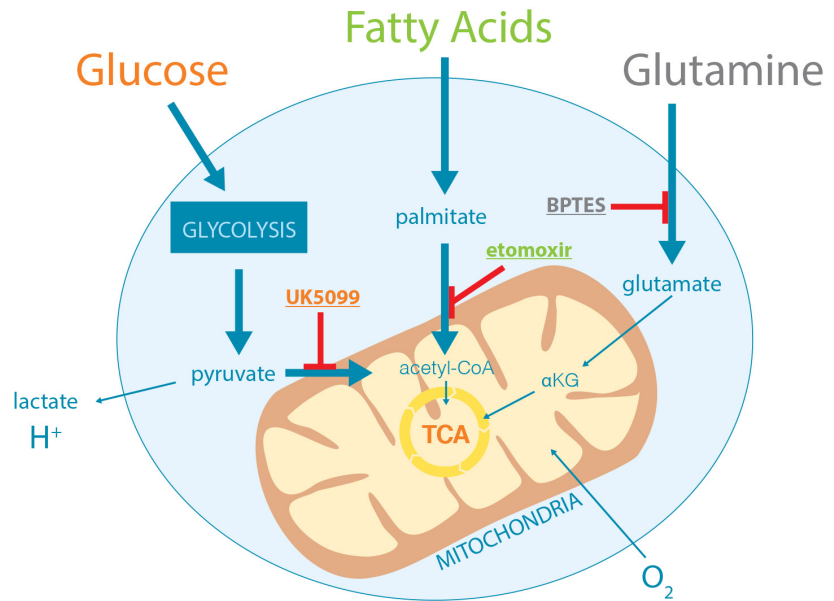
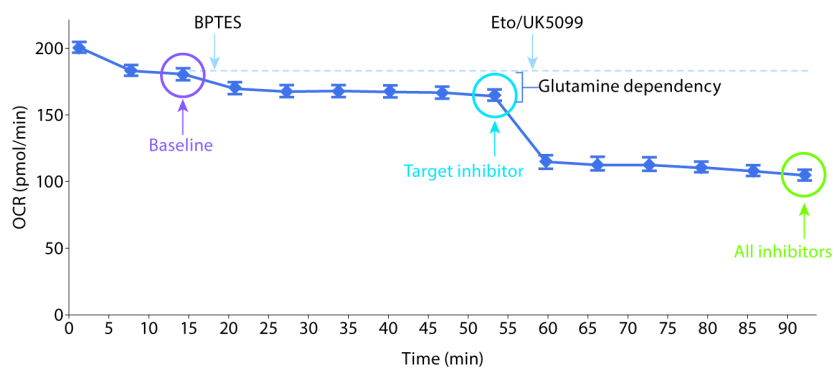
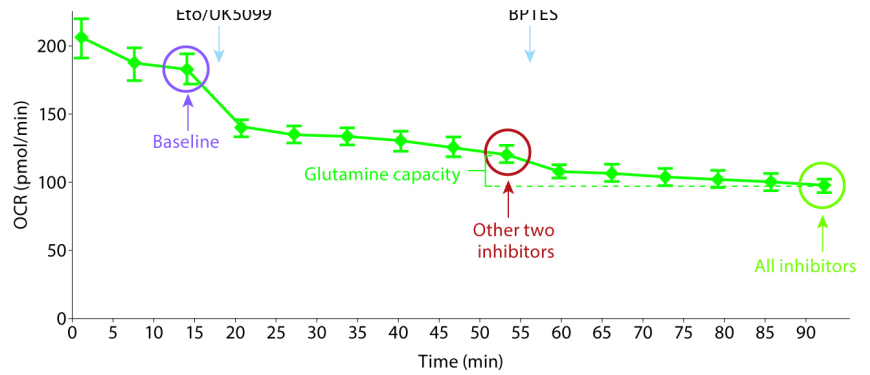


Figure 1 Principle of the Agilent Seahorse XF Mito Fuel Flex Test. Energy produced by cells can be derived from mitochondrial oxidation of glucose, glutamine, and fatty acids. The cells' mitochondrial dependency on and flexibility for each of these fuel sources is determined by measuring the decrease in fuel oxidation (decline in oxygen consumption rate) upon addition of one or more inhibitors.



$$\text{Dependency \%} = \left[\frac{\text{Baseline OCR} - \text{Target Inhibitor OCR}}{\text{Baseline OCR} - \text{All Inhibitors OCR}} \right] * 100$$

Figure 2 Fuel Dependency: Glutamine Oxidation Pathway Example
 Fuel Dependency is tested by first injecting an inhibitor of the target pathway, followed by inhibition of the two alternative pathways. Dependency is calculated using the equation shown here. In this example, HepG2 cells were tested for Glutamine Pathway Dependency (n=3). BPTES was injected following the third rate measurement ("Baseline" measurement). The sixth rate measurement following this injection is used for the calculation to allow sufficient time for the compound to have a complete effect ("Target Inhibitor" measurement). The final rate measurement ("All Inhibitors") is used to complete the equation.



$$\text{Capacity \%} = \left[1 - \left[\frac{\text{Baseline OCR} - \text{Other 2 Inhibitors OCR}}{\text{Baseline OCR} - \text{All Inhibitors OCR}} \right] \right] * 100$$

Figure 3 Fuel Capacity: Glutamine Oxidation Pathway Example
 Fuel Capacity is tested by first injecting inhibitors of the alternative pathways, followed by inhibition of the target pathway. Dependency is calculated using the equation shown here. In this example, HepG2 cells were tested for Glutamine Pathway Capacity (n=3). Eto and UK5099 were injected following the third rate measurement (“Baseline” measurement). The sixth rate measurement following this injection is used for the calculation to allow sufficient time for the compounds to have a complete effect (“Other 2 Inhibitors” measurement). The final rate measurement (“All Inhibitors”) is used to complete the equation.

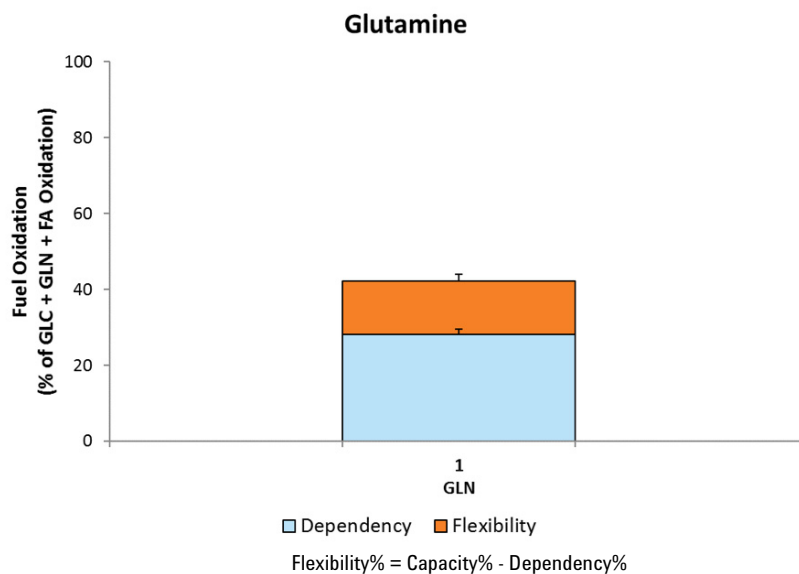


Figure 4 Fuel Flexibility: Glutamine Oxidation Pathway Example
Determination of Flexibility requires two groups: one Dependency group and one Capacity group (Figure 2 on page 8 and Figure 3 on page 9). Fuel Flexibility is calculated as the difference between Capacity and Dependency. All three parameters (Dependency, Capacity, and Flexibility) are displayed as a stacked bar chart when using the Agilent Seahorse XF Mito Fuel Flex Test Report Generator. See “[Data Analysis](#)” on page 19 for further information.

Glossary

Baseline Respiration Rate of oxygen consumption due to fuel oxidation under initial assay conditions.

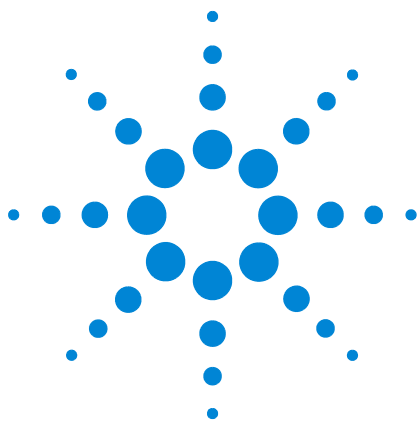
Fuel A substrate or nutrient that is used by cells and oxidized in the mitochondria. In this assay, the mitochondrial oxidation of glucose, glutamine, and/or long chain fatty acids is measured.

Fuel Pathway A series of biochemical processes that convert fuels into metabolites that are oxidized in the mitochondria (example: the conversion of glucose to pyruvate and transport of pyruvate into mitochondria).

Fuel Dependency The measurement of cells' reliance on a particular fuel pathway to maintain baseline respiration.

Fuel Capacity The ability of a cell's mitochondria to oxidize a fuel when other fuel pathways are inhibited.

Fuel Flexibility The difference between fuel capacity and dependency, that is, the ability of cells to increase oxidation of a particular fuel in order to compensate for inhibition of alternative fuel pathway(s).



2 Kit Information

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Kit Contents

The Agilent Seahorse XFp Mito Fuel Flex Test Kit contains sufficient compounds to complete six fuel dependency or combined dependency and flexibility tests (Figure 5 on page 13). The kit includes six foil pouches, each pouch contains one tube of each of the following compounds.

Table 1 Kit compounds

Compound	Cap color	Amount per tube (nmol)
BPTES	Grey	6
Etomoxir	Green	8
UK5099	Orange	4

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 product in users' hand varies from 12 to 3 months.



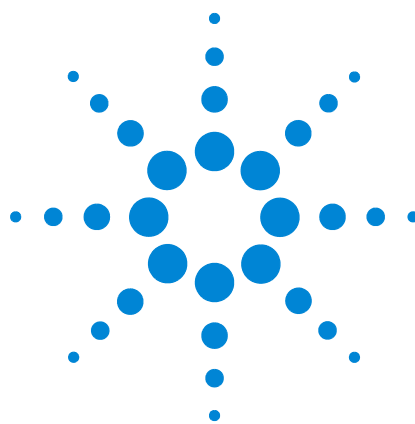
Additional Required Items

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

Table 2 Additional required items

Item	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
Microfuge tubes	Various	0.5-1.5 capacity

* 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 Workflow

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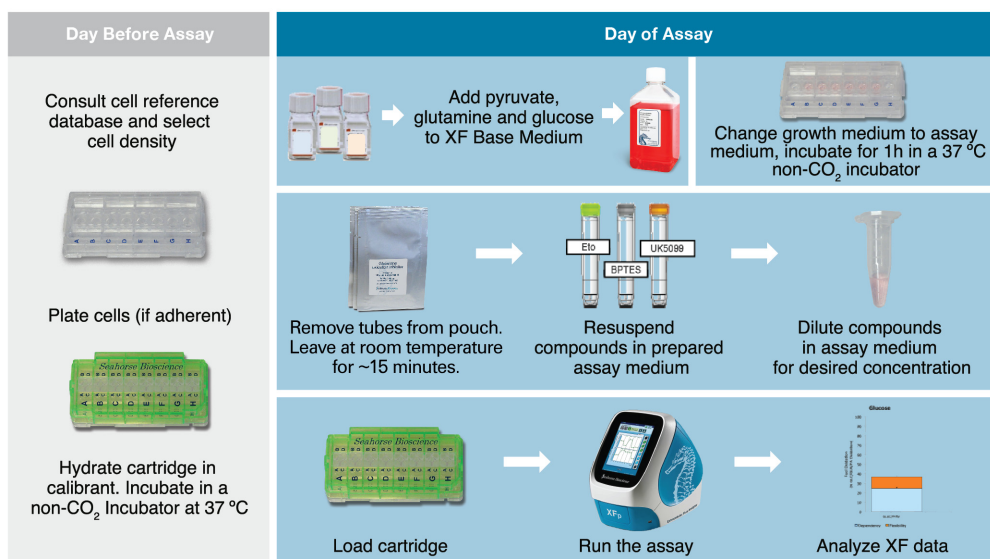


Figure 5 Seahorse XF Mito Fuel Flex Test Assay Workflow for the Agilent Seahorse XFp Analyzers

Day Prior to Assay

- 1 For adherent cells, plate cells at a previously determined density in the XFp Cell Culture Miniplate using the appropriate cell culture growth medium, adding medium only to wells A and H as well as the moats. Refer to Basic Procedures for the Agilent Seahorse XFp Analyzer:
<https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay>
- 2 Hydrate a sensor cartridge in Agilent Seahorse XF Calibrant at 37 °C in a non-CO₂ incubator overnight. Refer to Basic Procedures for the Agilent Seahorse XFp Analyzer.
- 3 Download the Seahorse XF Mito Fuel Flex Report Generator and both Dependency and Flexibility Assay Templates from the Agilent.com website. Seahorse recommends using the Assay Templates provided to perform the Dependency or Flexibility workflow. Both Assay Templates can be modified or customized using Wave Desktop.

<https://www.agilent.com/en/products/cell-analysis/cell-analysis-software/data-analysis/seahorse-xf-mito-fuel-flex-test-report-generator>

The Dependency or Flexibility assay may also be set up directly on the Agilent Seahorse XFp Analyzer. First, select the **Blank Template** then, after each injection, adjust the number of cycles to 6. See the Agilent Seahorse XFp Extracellular Flux Analyzer User Guide for additional details.

Day of Assay

- 1 Turn on the Agilent Seahorse XFp Analyzer and let it warm up (minimum 20 minutes).
- 2 Determine which type of assay you will run (dependency or flexibility), and which fuel pathway you will test (glucose, glutamine, or fatty acid). One Agilent Seahorse XFp Miniplate can accommodate one fuel assay: either a pairwise comparison of fuel dependency, or a single cell condition with both dependency and flexibility.

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 Agilent Seahorse XFp Cell Culture Miniplate for Assay

- Adherent cells
 - 1 Remove cell culture miniplate from 37 °C CO₂ incubator and examine cells under microscope to confirm that cells are 50-90 % confluent and evenly distributed within each well.
 - 2 Remove assay medium from water bath.
 - 3 Remove the cell culture growth medium in the cell culture miniplate and wash with warm assay medium. Remove the wash and add assay medium to total volume of 180 µL. Place the cell culture miniplate into a 37 °C non-CO₂ incubator for 1 hour prior to the assay.
- Suspension cells
 - 1 Harvest, count, and resuspend cells in assay medium at the desired density.
 - 2 Add 50 µL assay medium without cells to wells A and H of an Agilent Seahorse XFp Cell Culture Miniplate.
 - 3 Add 50 µL cells suspended in assay medium to wells B through G.

- 4 Centrifuge miniplate in Agilent Seahorse XFp Carrier Tray at $300 \times g$ for 1 minute.
- 5 Add 130 μL medium to each well to a total volume of 180 μL .
- 6 Place the miniplate into a 37 °C non-CO₂ incubator for 45 minutes prior to the assay.

Load template onto the Agilent Seahorse XFp Analyzer

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

- 1 Visit the Seahorse Bioscience website, and download the Agilent Seahorse XF Mito Fuel Flex Report Generator along with both Dependency and Flexibility Assay Templates. Both Seahorse XFp Mito Fuel Flex Test - Dependency and Seahorse XFp Mito Fuel Flex Test - Flexibility Assay Templates are included in the downloaded folder.
- 2 Copy the unzipped Assay Templates (*.asyt) files to the root directory of a USB Flash Drive.
- 3 Insert the USB drive into the front USB port of the Seahorse XFp Analyzer.
- 4 Click **Settings**, then click **Template Management**.
- 5 Click the **USB** tab, and locate the Assay Template(s) to upload.
- 6 Check the checkbox next to each Assay Template to be imported, then click **Import**.
- 7 All imported Assay Templates will now be available for selection from the **Local** tab when starting an XFp assay.

Removing reagent caps

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



Figure 6 Removing reagent cap

Prepare stock compounds

Important: Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound. Refer to “[Removing reagent caps](#)” on page 16 for instructions on removing the reagent caps.

- 1 Remove one foil pouch from Agilent Seahorse XFp Mito Fuel Flex Test Kit box.
- 2 Remove three tubes from the pouch, and place in a small tube rack.
- 3 Resuspend contents of each tube with prepared assay medium in volumes described in Table 2 with a pipette. Place cap on tube, and vortex for 1 minute to solubilize the compounds.

Table 3 Stock solution preparation

Compound name	Volume of assay medium (μL)	Stock concentration (μM)	10x Port concentration (μM)	Final assay well concentration (μM)
BPTES	100	60	30	3.0
Etomoxir	100	80	40	4.0
UK5099	100	40	20	2.0

Prepare compounds for loading in sensor cartridge ports

Chose ONE fuel pathway to assay then make the appropriate dilutions in a microfuge tube using [Table 4](#).

Table 4 Compound preparation for a dependency or flexibility test

Dilution plan (Choose ONE)	Tube	Contents	Volume BPTES (µL)	Volume Etomoxir (µL)	Volume UK5099 (µL)	Volume assay medium (µL)	Total volume (µL)
Glutamine oxidation	1	BPTES	90	-	-	90	180
	2	Etomoxir/UK5099	-	90	90	-	180
Fatty acid oxidation	1	Etomoxir	-	90	-	90	180
	2	BPTES/UK5099	90	-	90	-	180
Glucose oxidation	1	UK5099	-	-	90	90	180
	2	BPTES/Etomoxir	90	90	-	-	180

Load sensor cartridge

Load the sensor cartridge for either a Dependency or Flexibility assay using [Table 5](#).

NOTE

For a dependency assay, only the Dependency group is needed for the fuel of interest. (This allows for the second group to be a pairwise comparison.) For a flexibility assay, both Dependency and Capacity groups are needed for each fuel of interest.

Table 5 Compound loading

Assay group	Tube	Contents	Port	Volume
Glutamine dependency	1	BPTES	A	20 µL
	2	Etomoxir/UK5099	B	22 µL
Glutamine capacity	2	Etomoxir/UK5099	A	20 µL
	1	BPTES	B	22 µL
Fatty acid dependency	1	Etomoxir	A	20 µL
	2	BPTES/UK5099	B	22 µL
Fatty acid capacity	2	BPTES/UK5099	A	20 µL
	1	Etomoxir	B	22 µL
Glucose dependency	1	UK5099	A	20 µL
	2	BPTES/Etomoxir	B	22 µL
Glucose capacity	2	BPTES/Etomoxir	A	20 µL
	1	UK5099	B	22 µL

Running the Assay

- 1 Press **Start**.
- 2 Select the **Agilent Seahorse XFp Mito Fuel Flex Test - Dependency** or **Seahorse XFp Mito Fuel Flex Test - Flexibility Assay Template** from the **Local** tab.
- 3 Group Page: No action required - Confirm or adjust the groups and plate map, then press the right arrow.
- 4 Protocol Page: No action required - Confirm the protocol, then press the right arrow.
- 5 Summary Page: Change the name of the assay results file, if desired. Press **Start Assay** when ready.
- 6 Place the utility plate with the loaded assay cartridge on the instrument tray, and click **Continue**. Calibration will take approximately 20 minutes.
- 7 When calibration has been completed, remove the utility plate and place the Agilent Seahorse XFp Miniplate on the tray, and click **Continue** to start the assay.

Data Analysis

Of particular importance for the best quality data are items such as optimal cell density, consistent cell seeding (as reflected in CVs of absolute baseline rates among similarly treated wells), and stable baseline OCR values of the cell line/type being used in the assay. For all XF assays, significant variability between wells within the same group indicates the need for additional optimization of cell culture, cell seeding, or intervention/treatment conditions. If variability is high within a group, please review the Basic Procedures for your analyzer. For additional questions, please contact Technical Support.

After ensuring adequate data quality, proceed with data analysis using the Seahorse XF Mito Fuel Flex Test Report Generator. This report generator calculates the parameters of %Dependency, %Capacity, and %Flexibility with respect to each group or fuel tested. These parameters provide a relative comparison of mitochondrial fuel oxidation among groups during basal respiration conditions. It is also encouraged that absolute OCR values (in pmol/min) of control and experimental groups are reviewed for any significant changes in OCR baseline values between groups (measurement 3/measurement prior to any injection). These differences in OCR values between groups suggest some biological change, and should be incorporated into

the final interpretation of Mito Fuel Flex Test results.

Export to the Seahorse XF Mito Fuel Flex Test Report Generator using Wave Desktop to generate a one-page Summary Report. The Report Generator automatically calculates percent Dependency, Capacity, and Flexibility, providing a simple, standardized output for analysis and interpretation of Seahorse XF Mito Fuel Flex Test data, and supports data analysis from all Seahorse XF Analyzers.

To download the Seahorse XF Mito Fuel Flex Test Report Generator and accompanying user guide visit:

<https://www.agilent.com/en/products/cell-analysis/cell-analysis-software/data-analysis/seahorse-xf-mito-fuel-flex-test-report-generator>



4 Frequently Asked Questions

What if all three inhibitors only cause a small decrease in total OCR?

Processes other than oxidation of these three fuels may contribute to baseline OCR. These processes may be broken down further into mitochondrial and nonmitochondrial oxygen consuming processes:

Other mitochondrial respiration: respiration dependent on an alternative substrate(s) being oxidized to support mitochondrial respiration, which may include (but not limited to) short and medium chain fatty acids and amino acids other than glutamine.

Nonmitochondrial oxygen consumption: consumption of oxygen by other biochemical processes in the cell. This includes (but is not limited to) very long chain fatty acids that get partially oxidized in the peroxisomes and other cellular enzymatic processes that consume oxygen. The nonmitochondrial fraction of total oxygen consumption can be measured using the Seahorse XF Cell Mito Stress Test.

Why is Dependency reported as zero?

If Dependency is not significantly above zero or negative due to well to well variability, there is no dependency on that particular substrate. If the cells are not dependent on the target fuel pathway, OCR may slightly increase following injection of inhibitor. When this occurs, Dependency is automatically set to zero (no dependence), and Flexibility will be equal to Capacity.

What does it mean if I have negative flexibility values?

When changes in OCR are small, well to well variability might lead to negative flexibility values. Negative flexibility values of less than 5 % are generally attributable to noise in the assay. If you detect significant negative flexibility, contact Technical Support.



How can I further diagnose or troubleshoot Mito Fuel Flex Test results?

The above potential issues described (apparent low response to inhibitors, 0 % dependency, or negative flexibility values) may be further diagnosed by performing (as a separate test or added group) a Mito Fuel Flex Test with only media injections (no inhibitors) to establish if baseline respiration changes significantly over the course of the assay. If the absolute respiration rates (OCR) in the absence of inhibitors trend significantly upward or downward throughout the assay, then the parameters of this test (relative % dependency, capacity and flexibility) will be either underestimated or exaggerated, respectively. This depends on the magnitude and direction of any baseline trends versus the magnitude of change due to added inhibitors. To limit any upward/downward baseline trending OCR, ensure that cell culture and technical parameters of the assay have been thoroughly optimized.

Will these inhibitors and concentrations work with all cells?

Yes, the test uses all three compounds at concentrations well above their EC₅₀ values for inhibition in mammalian cells. These values have been validated in a variety of cell lines and primary isolates. While most cell types or cell lines have an appreciable response to at least one inhibitor, not all cells will respond to all inhibitors. If the cells are not responsive to a particular inhibitor, they may not be dependent on that particular fuel pathway (that is, they are flexible with respect to the fuel used for oxidative phosphorylation).

How do I interpret ECAR and glycolysis in this assay?

Using combinations of inhibitors can confound interpretation of ECAR data with this test due to shifts in cellular ATP production and demand. For directly measuring glycolytic function, we recommend using the Seahorse XF Glycolysis Stress Test.

The recommended assay medium does not include fatty acid, can I add it?

Although not required, long chain fatty acid may be added to the medium. We recommend using a single species of long chain fatty acid, such as Seahorse XF Palmitate-BSA FAO Substrate, when testing exogenous fatty acid oxidation. NOTE: only oxidation of long-chain fatty acid, such as palmitate, is sensitive to inhibition by etomoxir.



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