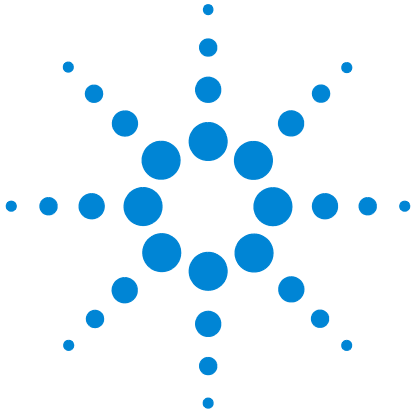

Agilent Seahorse XF Mito Fuel Flex Test Kit

For use with Agilent Seahorse XF Extracellular Flux Analyzers

User Manual
Kit 103260-100

Agilent Technologies

Notices

Manual Part Number

103260-400

Kit Part Number

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Contents

1 Introduction

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 XFe/XF96 or Agilent Seahorse XFe/XF24 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 using 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.

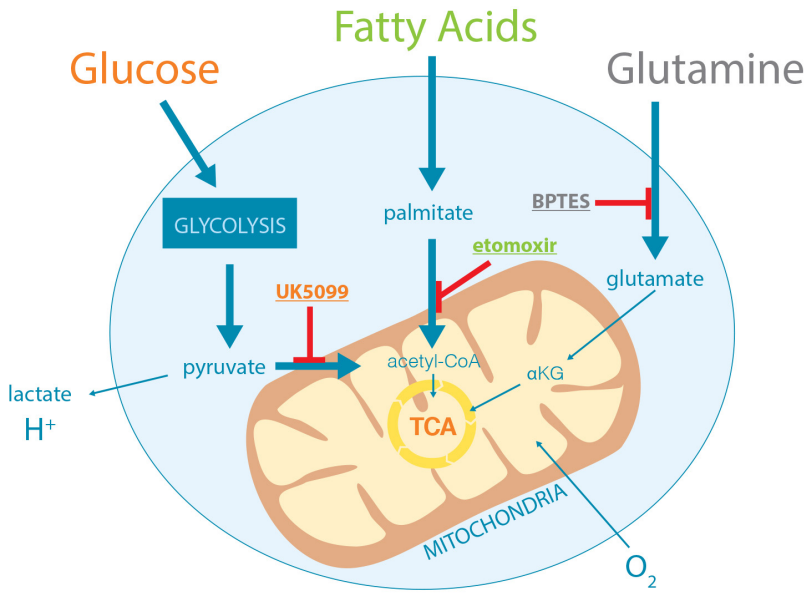

Figure 1 Principle of the Agilent Seahorse XF Mito Fuel Flex Test Energy produced by cells can be derived from mitochondrial oxidation of glucose, glutamine, and fatty acids. The cells' mitochondrial dependency on and flexibility for each of these fuel sources is determined by measuring the decrease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

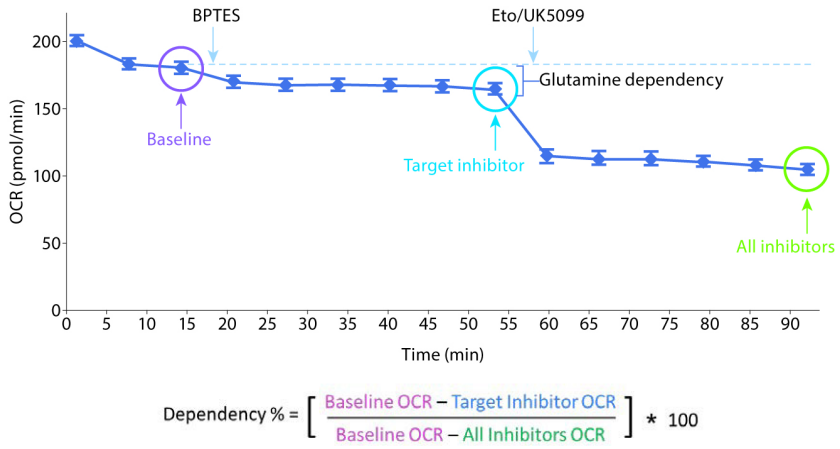

Figure 2 Fuel Dependency: Glutamine Oxidation Pathway Example
Fuel Dependency is tested by first injecting an inhibitor of the target pathway, followed by inhibition of the two alternative pathways. Dependency is calculated using the equation shown here. In this example, HepG2 cells were tested for Glutamine 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.

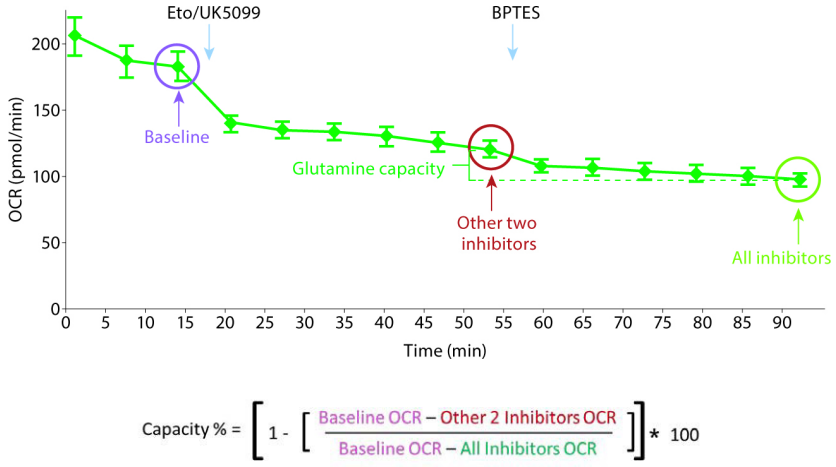

Figure 3 Fuel Capacity: Glutamine Oxidation Pathway Example
Fuel Capacity is tested by first injecting inhibitors of the alternative pathways, followed by inhibition of the target pathway. Dependency is calculated using the equation shown here. In this example, HepG2 cells were tested for Glutamine Pathway 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.

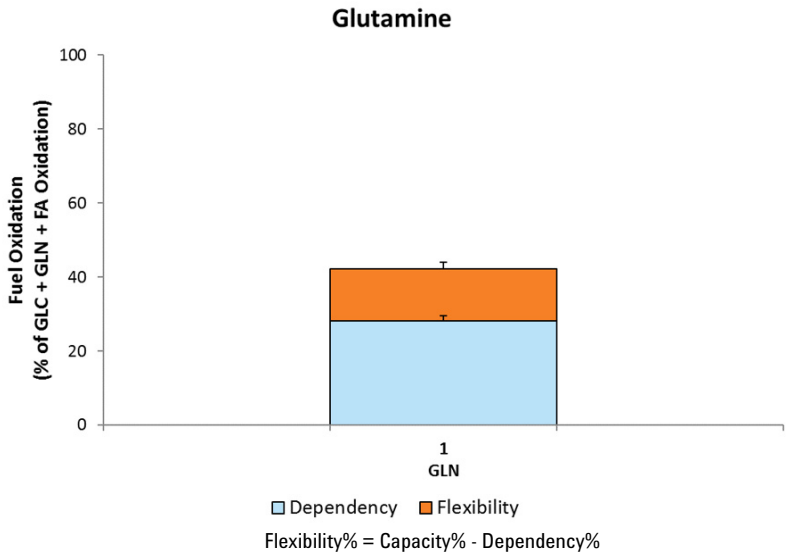

Figure 4 Fuel Flexibility: Glutamine Oxidation Pathway Example
Determination of Flexibility requires two groups: one Dependency group and one Capacity group (Figure 2 on page 8 and Figure 3 on page 9). Fuel Flexibility is calculated as the difference between Capacity and Dependency. All three parameters (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 21 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, 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 to compensate for inhibition of alternative fuel pathway(s).

2 Kit Information

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	84
Etomoxir	Green	112
UK5099	Orange	56

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 XFe/XF Analyzers	Agilent Technologies	
For XFe/XF96 Analyzers: XFe96 FluxPak mini or XFe96 FluxPak	Agilent Technologies	102601-100 or 102416-100
For XFe/XF24 Analyzers: XFe24 FluxPak mini or XFe24 FluxPak	Agilent Technologies	102342-100 or 102340-100
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
5-15 mL capacity tubes	Various	or reagent trough
Narrow p1000 pipette tips	Fisher Scientific	02-707-402

* 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

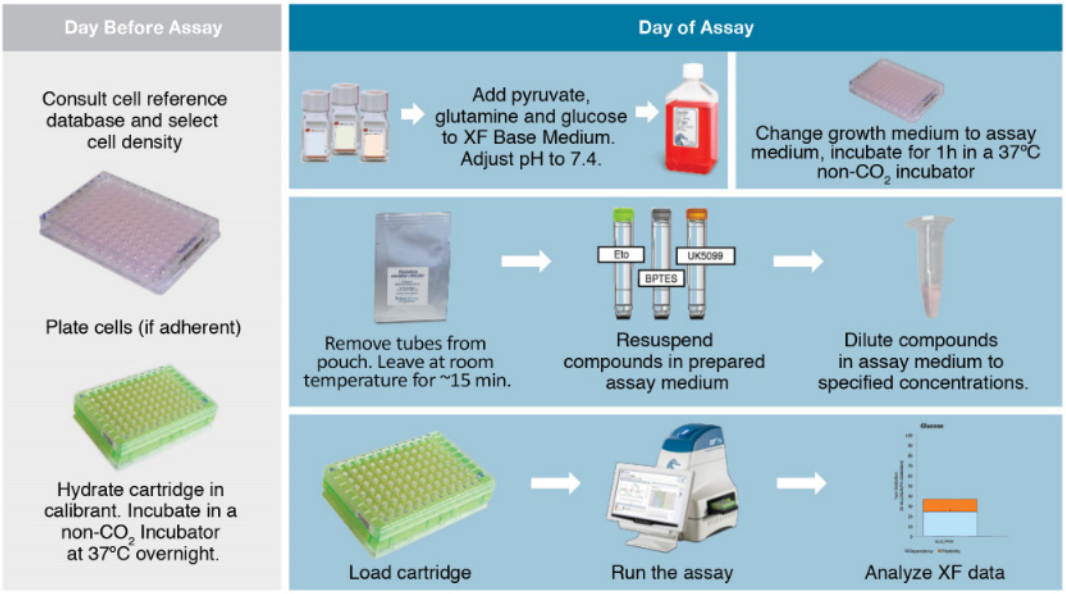

Figure 5 Agilent Seahorse XF Mito Fuel Flex Test Assay Workflow for the Agilent Seahorse XFe/XF Analyzers

Day prior to assay

1. Turn on the Agilent Seahorse XFe/XF Analyzer, and let it warm up overnight (minimum 5 hours).
2. Plate cells at a previously determined optimized density in the Agilent Seahorse XF Cell Culture Microplate using the appropriate cell culture growth medium. Refer to Basic Procedures for the Agilent Seahorse XF Analyzer at:

   https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay
3. Hydrate a sensor cartridge in Agilent Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight. Refer to Basic Procedures for the Agilent Seahorse XF Analyzer.
4. Access and customize the Dependency and Flexibility assay templates for the Seahorse XF Mito Fuel Flex Test using Wave Desktop. Import the customized assay template to the Agilent Seahorse XFe/XF Analyzer to run the assay. For instructions and to download Wave Desktop visit.

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

Day of 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 Agilent Seahorse XF Cell Culture Microplate for assay

1 Remove cell culture microplate from 37 °C CO_2 incubator and examine cells under microscope to confirm confluent.

2 Remove assay medium from water bath.

3 Remove the cell culture growth medium in the cell culture microplate, and wash with warm assay medium using a multichannel pipette. Remove the wash and add assay medium to total volume of 180 µL (for Agilent Seahorse XF96 Microplates) or 500 µL (for Agilent Seahorse XF24 Microplates). Place the cell culture microplate into a 37 °C non-CO_2 incubator for 1 hour prior to the 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.

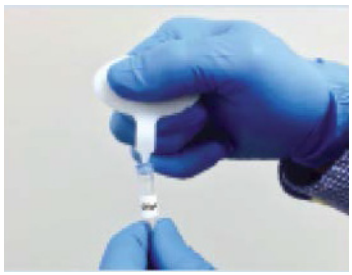

Figure 6 Removing reagent cap

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 15 for instructions on removing the reagent caps.

1 Remove one foil pouch from Agilent Seahorse XF Mito Fuel Flex Test Kit box.

2 Remove three tubes from the pouch, and place in a small tube rack.

3 Resuspend the contents of each tube with prepared assay medium in volumes described in Table 3 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	700	120	30	3.0
Etomoxir	700	160	40	4.0
UK5099	700	80	20	2.0

Prior to loading a sensor cartridge

Refer to the Basic Procedure for your analyzer for proper cartridge loading technique.

For each dependency assay, one group is needed for each fuel of interest.

For a Flexibility test, two groups are needed for each fuel of interest, one Dependency and one Capacity group.

Load compounds into the appropriate ports of a hydrated sensor cartridge (Refer to Tables 4-6). If using a template, follow the loading scheme provided on the **Plate Map** tab or redistribute groups appropriately (Refer to Figures 7-9).

Agilent Seahorse XFe/XF24: Compound preparation and sensor cartridge loading

Table 4 10x Compound preparation for a 24 well dependency test of all three fuel pathways
For a 24 well dependency test of a single pathway see Table 5.

Agilent Seahorse XFe/XF24	Tube label	Contents	Volume of BPTES (µL)	Volume of ETO (µL)	Volume of UK5099 (µL)	Volume of assay medium (µL)	Total volume (µL)
Glutamine oxidation	1	BPTES	220	X	X	660	880
	2	ETO/UK5099	X	220	220	440	880
Fatty acid oxidation	3	ETO	X	220	X	660	880
	4	BPTES/UK5099	220	X	220	440	880
Glucose oxidation	5	UK5099	X	X	220	660	880
	6	BPTES/ETO	220	220	X	440	880

Table 5 10x Compound preparation for a 24 well flexibility test (choose one fuel pathway)

Agilent Seahorse XFe/XF24	Tube label	Contents	Volume of BPTES (µL)	Volume of ETO (µL)	Volume of UK5099 (µL)	Volume of assay medium (µL)	Total volume (µL)
Glutamine oxidation	1	BPTES	700	X	X	2100	2800
	2	ETO/UK5099	X	700	700	1400	2800
Fatty acid oxidation	3	ETO	X	700	X	2100	2800
	4	BPTES/UK5099	700	X	700	1400	2800
Glucose oxidation	5	UK5099	X	X	700	2100	2800
	6	BPTES/ETO	700	700	X	1400	2800

Table 6 Loading Sensor Cartridge. For a Flexibility test run both Dependency and Capacity tests

Agilent Seahorse XFe/XF24	Tube label	Contents	Port	XFe/XF24
Glutamine dependency	1	BPTES	A	56 µL
	2	ETO/ UK5099	B	62 µL
Glutamine capacity	2	ETO/ UK5099	A	56 µL
	1	BPTES	B	62 µL
Fatty acid dependency	3	ETO	A	56 µL
	4	BPTES/ UK5099	B	62 µL
Fatty acid capacity	4	BPTES/ UK5099	A	56 µL
	3	ETO	B	62 µL
Glucose dependency	5	UK5099	A	56 µL
	6	BPTES/ ETO	B	62 µL
Glucose capacity	6	BPTES/ ETO	A	56 µL
	5	UK5099	B	62 µL

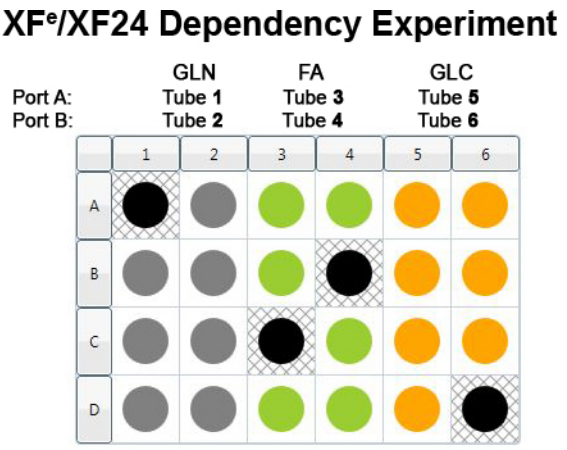

Figure 7 Agilent Seahorse XFe/XF24 Dependency test plate map

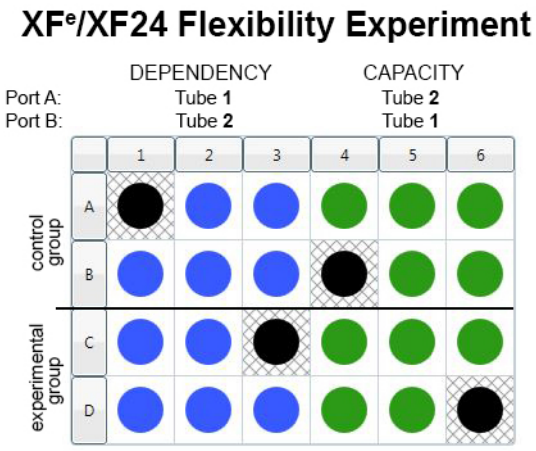

Figure 8 Agilent Seahorse XFe/XF24 Flexibility test plate map

Agilent Seahorse XFe/XF96: Compound preparation and sensor cartridge loading

Table 7 10x Compound preparation for a 96 well dependency or flexibility test

Agilent Seahorse XFe/XF96	Tube label	Contents	Volume of BPTES (µL)	Volume of ETO (µL)	Volume of UK5099 (µL)	Volume of assay medium (µL)	Total volume (µL)
Glutamine oxidation	1	BPTES	220	X	X	660	880
	2	ETO/UK5099	X	220	220	440	880
Fatty acid oxidation	3	ETO	X	220	X	660	880
	4	BPTES/UK5099	220	X	220	440	880
Glucose oxidation	5	UK5099	X	X	220	660	880
	6	BPTES/ETO	220	220	X	440	880

Table 8 Loading sensor cartridge for a flexibility test perform both dependency and capacity tests

AgieInt Seahorse XFe/XF96	Tube label	Contents	Port	Agilent Seahorse XFe/XF96
Glutamine dependency	1	BPTES	A	20 µL
	2	ETO/ UK5099	B	22 µL
Glutamine capacity	2	ETO/ UK5099	A	20 µL
	1	BPTES	B	22 µL
Fatty acid dependency	3	ETO	A	20 µL
	4	BPTES/ UK5099	B	22 µL
Fatty acid capacity	4	BPTES/ UK5099	A	20 µL
	3	ETO	B	22 µL
Glucose dependency	5	UK5099	A	20 µL
	6	BPTES/ ETO	B	22 µL
Glucose capacity	6	BPTES/ ETO	A	20 µL
	5	UK5099	B	22 µL

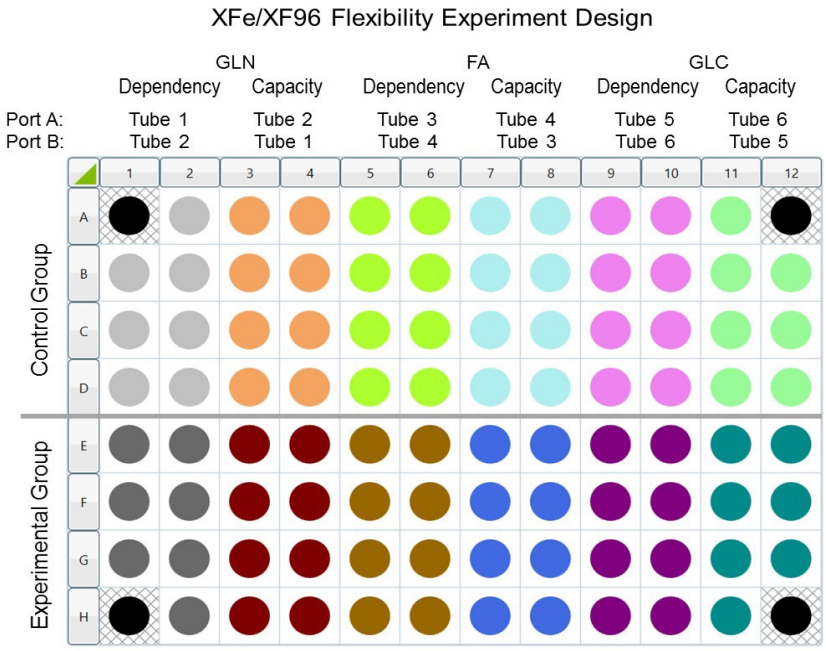

Figure 9 Agilent Seahorse XFe/XF96 Flexibility test plate map

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. Open Wave Desktop 2.3.
2. Select the Seahorse XF Mito Fuel Flex Template(s) to export and click **Export**.
3. Save the assay template(s) to a USB flash drive or network drive (if Agilent Seahorse XFe Analyzer is networked).

Seahorse Agilent XFe96/XFe24 Analyzer:

1. Insert the USB flash drive to the front USB port on the XFe Controller and wait ~10 seconds.
2. Press **Import** (bottom of the New view).
3. Locate the assay template(s) on the USB flash drive or network drive and click **Open**.
   - Repeat for next assay template (if applicable).

4 The imported assay template(s) are now available for selection on the New view on Wave Controller.

Run the Seahorse XF Mito Fuel Flex Test Assay Template:

1 Double-click the **Dependency** or **Flexibility** assay template from the list of available templates (or select the template and click **Design**).

2 Groups Definitions Tab - No action required. Add or modify groups and conditions for the assay.

3 Plate Map Tab - No action required. Modify the Plate Map to select assay wells for each group.

4 Instrument Protocol Tab - No action required. Modify the Instrument Protocol to add additional measurement cycles.

5 Review and Run Tab - Press **Start Run** when ready.

6 When prompted, place the loaded sensor cartridge with the calibrant plate into the Agilent Seahorse XFe Analyzer, then click **I'm Ready**. Calibration will take approximately 15-30 minutes.

NOTE Remove cartridge lid and verify correct plate orientation.

7 Click **I'm Ready** after calibration to load the cell culture microplate.

8 Click **I'm Ready** to close the tray door and begin 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 Agilent 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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