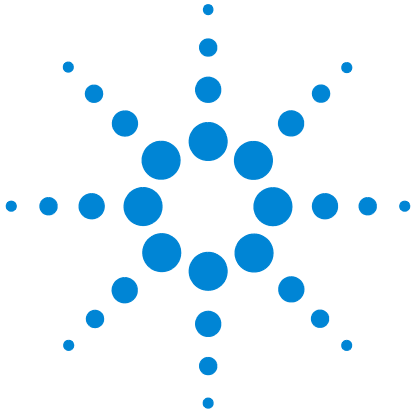

Agilent Seahorse XF Mito Fuel Flex Test Kit

User Guide

Agilent Technologies

Notices

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Contents

1 Introduction

The Agilent Seahorse XF Mito Fuel Flex Test is a comprehensive 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 critical mitochondrial fuels:

- Glucose
- Glutamine
- 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). Initially inhibiting one pathway measures how dependent the cells are on that fuel source to meet energy demand (Figure 2 on page 7). Dependency indicates that the cells are unable to compensate for the blocked pathway by utilizing other fuel pathways. Using a combination of inhibitors measures cells' capacity and flexibility in meeting energy demand. Flexibility indicates the cells have the ability to compensate for the inhibited pathway by utilizing other pathways to fuel mitochondrial respiration (Figure 3 on page 7). The Seahorse XF Mito Fuel Flex Test Kit contains the 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 utilized 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, which is then converted to alpha-ketoglutarate and directly utilized by the TCA cycle
- **Etomoxir** - An inhibitor of the long chain fatty acid oxidation pathway. 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.

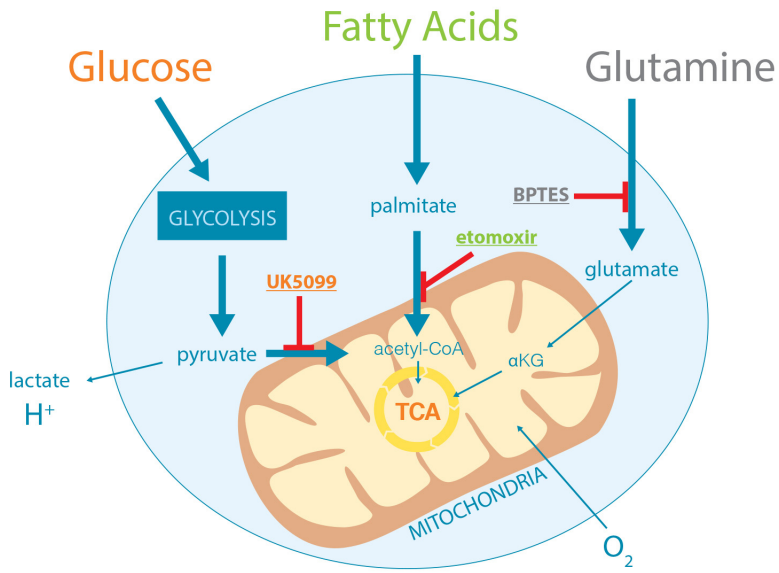

Figure 1 Principle of the Agilent Seahorse XF Mito Fuel Flex Test

The majority of energy produced by cells is derived from three fuels: glucose, glutamine, and fatty acids. The cells' dependency on, and flexibility for using each of these fuel sources is determined by measuring the decrease in fuel oxidation (decline in oxygen consumption rate) upon the addition of one or more inhibitors.

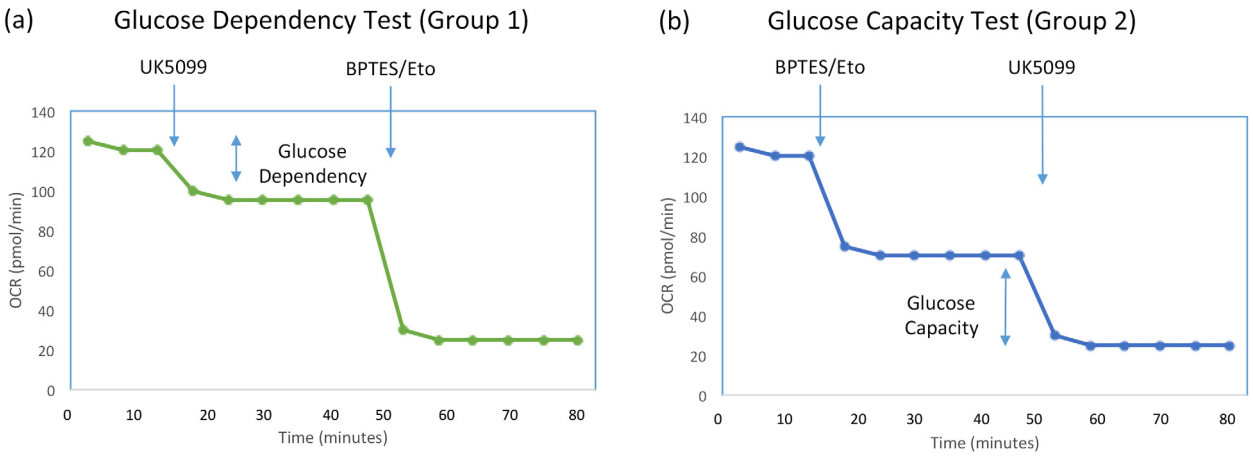

Figure 2 Fuel Dependency and Capacity: Glucose Oxidation Pathway Example

Fuel dependency, Figure 2 (a), is determined by first injecting an inhibitor of the target pathway, followed by inhibition of the two alternative pathways. Fuel capacity, Figure 2 (b), is determined by inhibiting the two alternative pathways first, followed by the target pathway.

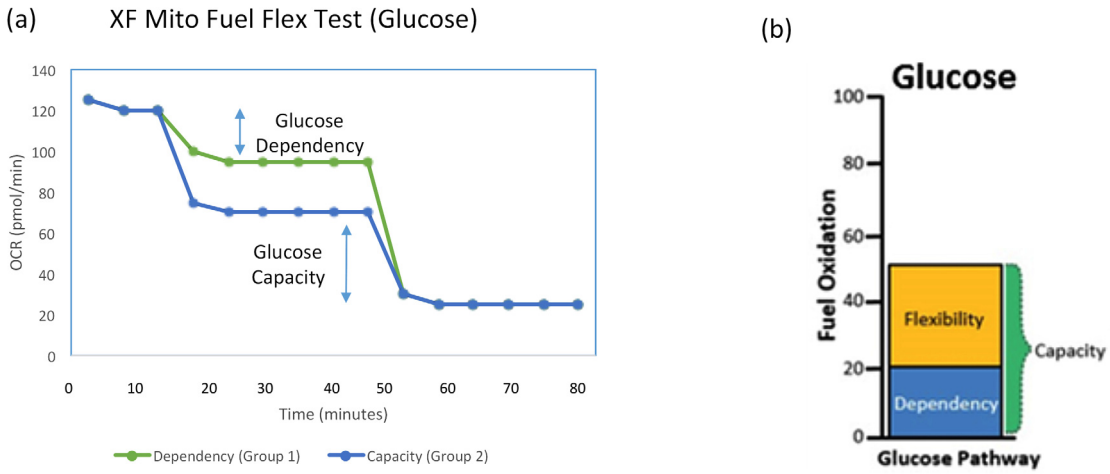

Figure 3 Fuel Flexibility: Glucose Oxidation Pathway Example

Determination of flexibility requires both groups from Figure 1. Fuel flexibility is calculated as the difference between capacity and dependency Figure 3 (a). 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 Figure 3 (b). (See Data Analysis for further information.)

Kit Contents

The Seahorse XF Mito Fuel Flex Test Kit contains sufficient compounds to complete six fuel dependency or combined dependency and flexibility tests (Figure 4 on page 11). The kit includes three foil pouches - one for each of the three mitochondrial fuel inhibitors. Each pouch contains six tubes of each of the following compounds.

Table 1 Kit Compounds

Compound	Cap color	Amount per tube (nmol)
BPTES	Grey	84
Etomoxir	Green	1120
UK5099	Orange	56

Kit Shipping and Storage

Product ships at ambient temperature in an insulated cooler box. Product should be stored at -20 °C for up to 1 year from the date of manufacture (listed on package).

Additional required items	Supplier	Part number
Agilent Seahorse XFe/XF Analyzer	Agilent Seahorse Bioscience	
Agilent Seahorse XF Base Medium	Agilent Seahorse Bioscience	102353-100
Agilent Seahorse XFe/XF FluxPak	Agilent Seahorse Bioscience	
100 mM Pyruvate	Sigma	S8636 or equivalent
200 mM Glutamine	Sigma	G8540 or equivalent
2.5 M Glucose	Sigma	G8769 or equivalent
Microfuge tubes	Various	0.5-1.5 mL capacity
Narrow p1000 pipette tips 5-15 mL capacity tubes	Fisher Scientific Various	02-707-402 (SureOne™ Micropoint) or reagent trough

Glossary

- **Baseline respiration:** Rate of oxygen consumption required for fuel oxidation under initial assay conditions.
- **Fuel:** A substrate or nutrient that is utilized by cells and oxidized in the mitochondria. In this assay, the fuel is glucose, glutamine, or long chain fatty acids.
- **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 cells' ability to use a fuel pathway to meet energy demand when other fuel pathways are inhibited.
- **Fuel flexibility:** The ability of cells to increase oxidation of a particular fuel to compensate for inhibition of alternative fuel pathway(s).

2 Assay Workflow

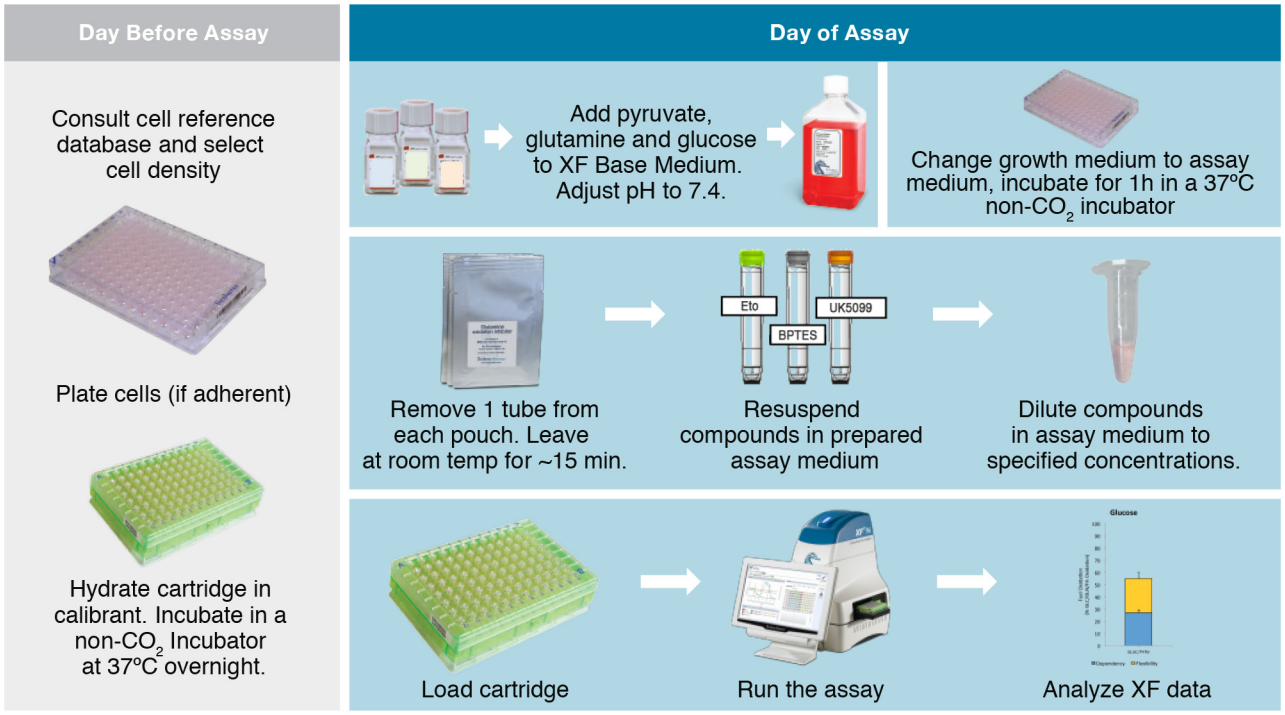

Figure 4 Agilent Seahorse XF Mito Fuel Flex Test assay workflow for the Agilent Seahorse XFe/XF Analyzers

Day Prior to Assay

1. Turn on the Seahorse XFe/XF Analyzer, and let it warm up overnight (minimum 5 hours).
2. Plate the 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 Procedure "Seeding Cells in XF Cell Culture Microplates" available on the Agilent Seahorse Bioscience website for more information.
   www.seahorsebio.com/resources/documentation/basicprocedures
3. Hydrate a sensor cartridge in Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight. Refer to Basic Procedure "Hydrating the Sensor Cartridge" on the Seahorse website for more details.
   www.seahorsebio.com/resources/documentation/basicprocedures
4. Download the Seahorse XF Mito Fuel Flex Report Generator and the appropriate Dependency and Flexibility Assay Templates (for Seahorse XFe Analyzers) from the Agilent Seahorse Bioscience Website. Seahorse Bioscience recommends that Agilent Seahorse XFe Analyzer users load the Assay Templates provided. Both Assay Templates can be modified or customized for your assay using a Wave Desktop. See instructions on loading the Assay Templates onto your Seahorse XFe Analyzer.
   www.seahorsebio.com/resources/reportgenerators/mitofuelflextest

This assay may also be designed on your Seahorse XFe Analyzer. Refer to the XFe Wave User Guide, available on the Seahorse Bioscience website.
www.seahorsebio.com/support/software/PDF/XFe_Wave_2_2_User_Guide_Final.pdf

Day of the Assay

Prepare assay medium

1. Prepare 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, 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 Seahorse XF Assays" on the Seahorse Bioscience website for more information. www.seahorsebio.com/resources/documentation/basicprocedures
2. Warm tha 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.

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 5 on page 13.

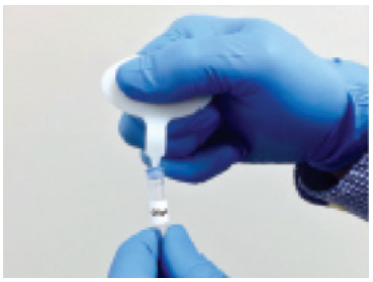

Figure 5 Removing reagent caps

Prepare Agilent Seahorse XF Cell Culture Microplate for assay

1. Remove the cell culture microplates from the 37 °C CO_2 incubator and examine cells under a microscope to confirm confluence.
2. Remove the assay medium from the 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 adjust the volume to 180 L (for Seahorse XFe/XF96), or 500 L (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 the Basic Procedure, "Washing Cells in XF Cell Culture Microplates" on the Seahorse Bioscience website for proper cell washing technique.
http://www.seahorsebio.com/resources/documentation/basicprocedures/

Prepare stock compounds

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

Note: Refer to "Removing reagent caps" on page 13 for instructions on removing the reagent caps.

1 Remove all three foil pouches from the Seahorse XF Mito Fuel Flex Test Kit box. Remove one tube from each pouch. Tightly reseal pouches, and immediately return to -20 °C.

2 Place tubes in a small tube rack. Allow compounds to warm to room temp for approximately 15 minutes.

3 Using a pipette, resuspend the contents of each tube with prepared assay medium in volumes described in Table 2. Place a cap on the tube, and vortex for 1 minute to solubilize the compounds.

Table 2 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	1600	40	4.0
UK5099	700	80	20	2.0

Prior to loading a sensor cartridge

Refer to the Basic Procedure, "Loading the Sensor Cartridge with Compounds" on the Seahorse Bioscience website for proper port-loading technique.
http://www.seahorsebio.com/resources/documentation/basicprocedures/

Note: For each dependency assay, one group is needed for each fuel of interest. For each flexibility assay, two groups are needed for each fuel of interest.

Load compounds into the appropriate ports of a hydrated sensor cartridge (Refer to Table 3 on page 15, Table 4 on page 15, and Table 5 on page 16). If using a template, follow the loading scheme provided on the 'Plate Map' tab or redistribute groups appropriately (Refer to Figure 6 on page 17, Figure 7 on page 17, and Figure 8 on page 19).

Prepare compounds for loading sensor cartridge Seahorse XFe/XF24

For a 24 well dependency test of a single pathway, use Table 4.

Table 3 10x Compound Preparation for a 24 well dependency test of all three pathways

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 Dependency	1	BPTES	220	x	x	660	880
	2	ETO/ UK5099	X	22	220	638	880
Fatty Acid Dependency	3	ETO	X	22	x	858	880
	4	BPTES/ UK5099	220	x	220	440	880
Glucose Dependency	5	UK5099	X	x	220	660	880
	6	BPTES/ ETO	220	22	x	638	880

Table 4 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 Flexibility	1	BPTES	700	X	x	2100	2800
	2	ETO/ UK5099	X	70	700	2030	2800
Fatty Acid Flexibility	3	ETO	X	70	x	2730	2800

Table 4 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)
	4	BPTES/ UK5099	700	X	700	1400	2800
Glucose Flexibility	5	UK5099	X	X	700	2100	2800
	6	BPTES/ ETO	700	70	x	2030	2800

Table 5 Loading Sensor Cartridge. For a flexibility test run both Dependency and Capacity assay groups

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

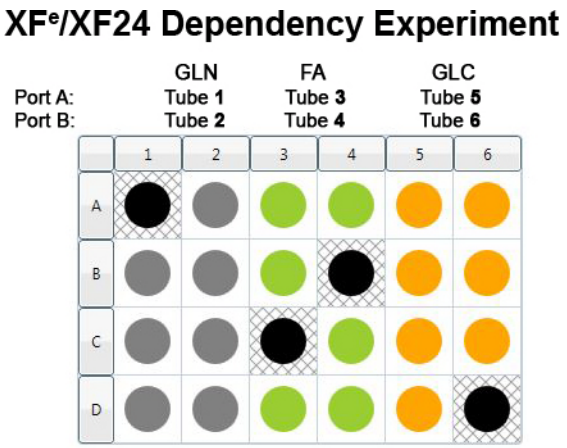

Figure 6 Agilent Seahorse XFe/XF24 dependency assay plate map

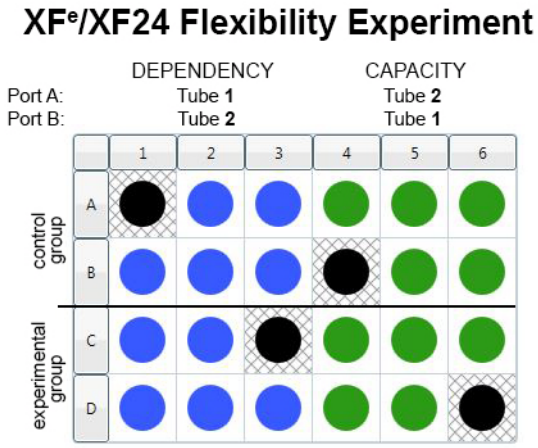

Figure 7 Agilent Seahorse XFe/XF24 flexibility assay plate map

Prepare compounds for loading sensor cartridge Seahorse XFe/XF96

For a single pathway, triple all volumes for the pathway of interest.

Table 6 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	22	220	638	880
Fatty Acid Oxidation	3	ETO	x	22	x	858	880
	4	BPTES/ UK5099	220	X	220	440	880
Glucose Oxidation	5	UK5099	x	X	220	660	880
	6	BPTES/ ETO	220	22	x	638	880

Table 7 Loading Sensor Cartridge. For a flexibility test perform both Dependency and Capacity

Agilent Seahorse XFe/XF96	Tube label	Contents	Port	Agilent Seahorse XFe/XF 96
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

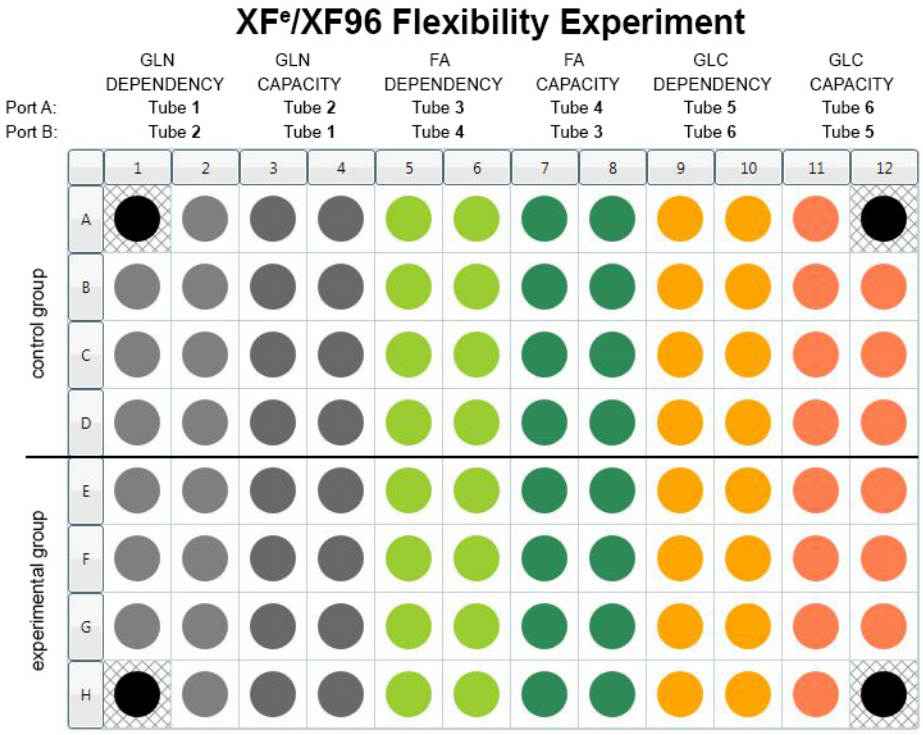

Figure 8 Agilent Seahorse XFe/XF96 Flexibility Assay Plate Map

Run 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 Mito Fuel Flex Report Generator from the Seahorse Bioscience website. Both the **Seahorse XF Mito Fuel Flex Test - Dependency** and the **Seahorse XF Mito Fuel Flex Test - Flexibility** Assay Templates are included in the downloaded folder.

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

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

Seahorse XFe96/XFe24 Analyzer:

1. Insert the USB drive in the front USB port and wait ~10 seconds.
2. Click **Import** (bottom of the New Assay view).
3. Locate the Assay Template to import on the USB or Network drive.
4. Click **Open** in the Windows dialogue box, and repeat for next template, if applicable.
5. The imported Assay Template(s) will be available for selection in the list of available templates.

Run the Seahorse XF Mito Fuel Flex Test

1. Select the **Seahorse XF Mito Fuel Flex Test - Dependency** or **Seahorse XF Mito Fuel Flex Test - Flexibility** Assay Template from the list of available templates and press 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 measurements cycles during the assay.

- **Review and Run:** Click **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 takes approximately 15-30 minutes. **Note**: Remove the cartridge lid, and verify correct plate orientation

3 Click **I'm Ready** after Calibration to load the cell culture microplate.

4 Click **I'm Ready** to close the tray door and begin the assay.

Data Analysis

The Seahorse XF Mito Fuel Flex Test Report Generator is a critical component of the Seahorse XF Mito Fuel Flex Test. This Excel macro-based analytical tool automatically calculates percent Dependency and Flexibility, and provides a standardized output for analysis and interpretation of XF Seahorse Mito Fuel Flex Test data. The Seahorse XF Mito Fuel Flex Test Report Generator supports both Dependency and Flexibility assay workflows, and provides a convenient, customizable, one-page assay summary.

To download the Seahorse XF Mito Fuel Flex Test Report Generator and accompanying user guide visit: www.seahorsebio.com/support/software/report-generators.php

3 Frequently Asked Questions

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 is sensitive to inhibition by etomoxir.

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 at: support@seahorsebio.com.

Why is Dependency reported as zero?

If Dependency is not significantly above zero, it may be concluded that 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 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 non-mitochondrial 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.

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.

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 energy production).

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