Agilent

Agilent XF Substrate Oxidation Stress Test Kits

User Manual

XF Long Chain Fatty Acid Oxidation Stress Test Kit (103672-100)
XF Glucose/Pyruvate Oxidation Stress Test Kit (103673-100)
XF Glutamine Oxidation Stress Test Kit (103674-100)
XF Palmitate Oxidation Stress Test Kit (103693-100)

Notices

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Contents

1 Introduction

Assay Background

Agilent Seahorse XF technology measures energy metabolism in live cells in real time, providing critical functional information that relates directly to cellular health and fitness. The Seahorse XF Substrate Oxidation Stress Test Assay provides key metrics to facilitate the assessment of specific mitochondrial substrates that are relevant or required for cellular phenotype and function.

Agilent provides a suite of optimized, rapid solutions for measuring cellular substrate oxidation by assessing changes in oxygen consumption (OCR) using live cells. XF Substrate Oxidation Stress Test Kits allow for both a sensitive measure of mitochondrial function, and the interrogation of three primary substrates that fuel mitochondria: long-chain fatty acids (LCFAs), glucose/pyruvate (G/P), and/or glutamine (Q) (**Figure 1**). These kits facilitate the convenient investigation of specific substrate oxidation processes and the central role they play in the fundamental cellular functions of activation, proliferation, and differentiation; as well as in better characterizing cellular responses to genetic manipulation, pharmaceutical interventions, or specific disease-relevant microenvironments.

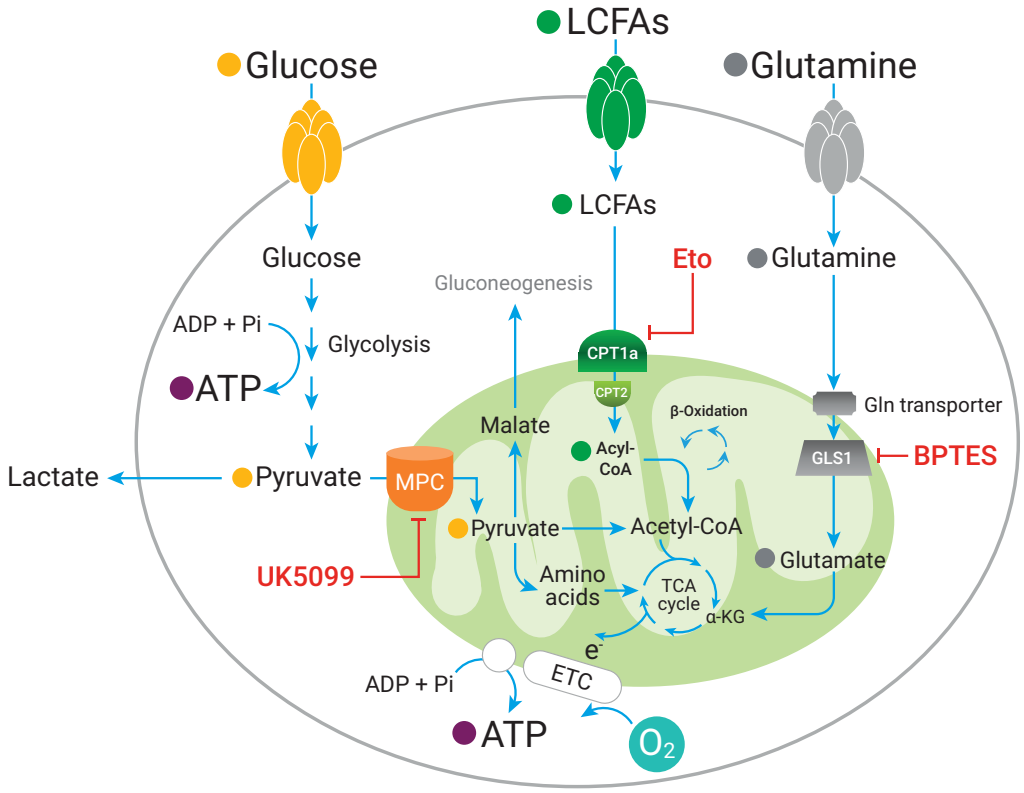


Figure 1. Primary metabolic pathways, including glycolysis, the TCA cycle, electron transport (ETC), and oxidative phosphorylation (OXPHOS). Glucose/pyruvate, glutamine and long chain fatty acid oxidation are highlighted. Red lines denote relevant inhibitors of glucose/pyruvate, LCFA, and glutamine transport, which in turn, specifically limits oxidation of that respective substrate.

The XF Substrate Oxidation Stress Tests combine the substrate pathway specific inhibitors (**Figure 1**): etomoxir for LCFAs through inhibition of carnitine palmitoyl transferase 1a (CPT1a)[1]; UK5099 for glucose and/or pyruvate through inhibition of the mitochondrial pyruvate carrier (MPC)[2]; and BPTES for inhibition of glutamine through glutaminase 1 (GLS-1)[3], with the XF Cell Mito Stress Test (MST). The MST, a powerful and well-accepted tool for the interrogation of mitochondrial function, in conjunction with these inhibitors, can be used to reveal dependence on a specific metabolic substrate. Basal and maximal respiration rates are key metrics of

mitochondrial function reported by the MST. In the context of substrate oxidation, the basal, and in particular, the maximal respiration rates are largely impacted by cells capacity to transport and oxidize available substrates.[4] This method is ideally suited to the assessment for cellular substrate demand both under basal conditions, and in response to elevated substrate demand (maximal respiration). **Figure 2** outlines the kinetic profile of a standard substrate oxidation assay and relevant assay parameters.

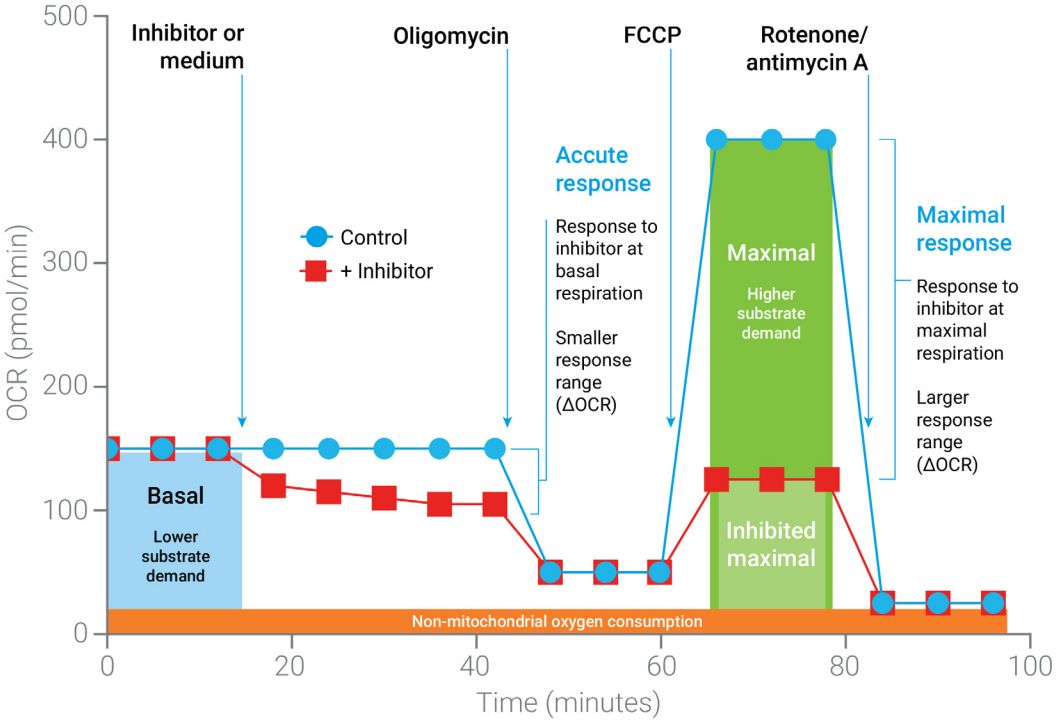


Figure 2. Agilent Seahorse XF Substrate Oxidation Stress Test profile of the respiration parameters critical for substrate demand. Sequential compound injections measure basal respiration, acute response to an inhibitor (etomoxir or UK5099 or BPTES), and maximal respiration in the absence and presence of inhibitor. Note that while minimal changes may be measured under basal conditions, such as the acute response; much larger responses are often revealed under conditions of high substrate demand (for example, FCCP), thus revealing differences in the ability of the cells to oxidize the substrate in question.

Measurements are highly informative, as the design described provides information on basal respiration and the impact of pathway inhibition under conditions of basal substrate demand, while also characterizing the impact of pathway inhibitions on maximal respiration, reflecting the cell's sensitivity to impairment of a specific metabolic pathway under conditions of high substrate demand. Due to the metabolic plasticity of many cell types, it is often more informative to assess substrate dependence when demand is high as this reveals the cell's capacity to use substrates. When mitochondria are uncoupled (such as FCCP exposure), substrate oxidation is increased to generate the reducing equivalents needed for the increased respiration rate. By comparing the maximum respiration rates of control versus inhibited cells, insights can be gained into how much a cell will rely on a specific substrate to meet this high energy demand.

This manual details how to perform all assays in the XF Substrate Oxidation Stress Test suite. In general, there are two distinct assay types: Standard and Advanced. The XF LCFA Oxidation Stress Test, XF Glutamine Oxidation Stress Test and Glucose/Pyruvate Oxidation Stress Test kits are to be carried out using the Standard Assay workflow, using a single inhibitor in the presence of multiple substrates). Conversely, the XF Palmitate Oxidation Stress Test Kit requires users to use the Advanced Assay protocol as additional steps are required (Figures **5**, **6**, and **10**), and is carried out using palmitate as the long chain fatty acid substrate and etomoxir as the inhibitor. The following are the principles and designs of these assays.

XF Substrate Oxidation Stress Test - Standard Assay

Relevant kits include:

XF Long Chain Fatty Acid Oxidation Stress Test Kit	(p/n 103672-100)
XF Glucose/Pyruvate Oxidation Stress Test Kit	(p/n 103673-100)
XF Glutamine Oxidation Stress Test Kit	(p/n 103674-100)

To perform a Standard Substrate Oxidation Stress Test, basal respiration is first established, followed by injection of the relevant pathway inhibitor. The acute response to the inhibitor is monitored over several measurement cycles (typically six). Then, the standard XF Cell Mito Stress Test reagents, such as oligomycin, FCCP, and rotenone/antimycin A are injected sequentially, similar to the assay scheme used for the XF Cell Mito Stress Test. **Figure 3** outlines an overview of the experimental workflow for each kit.

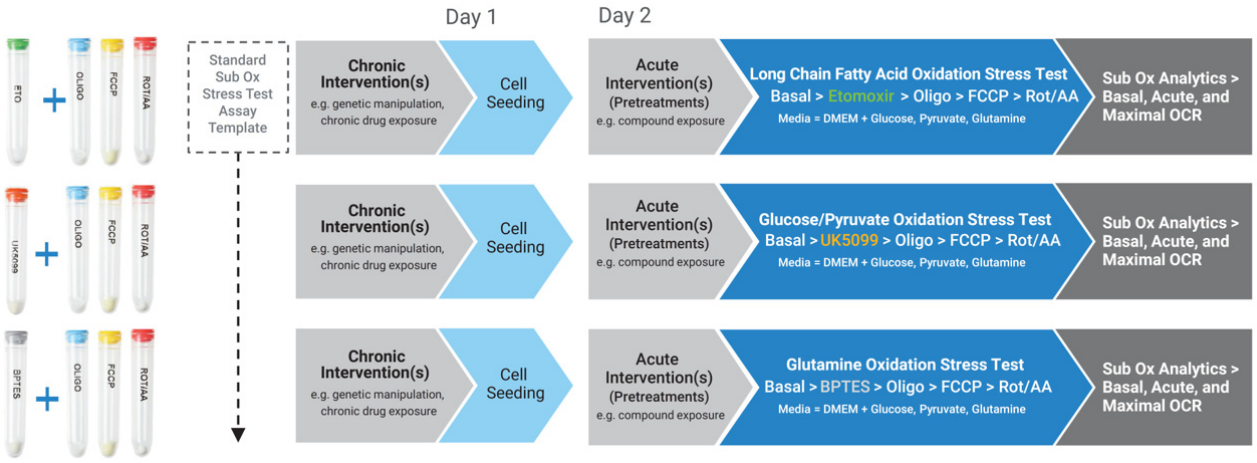


Figure 3. Agilent XF Substrate Oxidation Stress Test - Standard Assay Design. Each kit is focused on testing a single substrate using the relevant inhibitor. Chronic or acute interventions (genetic manipulations/drug exposure) may be performed upstream of the assay to understand the effects of these modulations on the oxidation of specific mitochondrial substrates.

Each kit is focused on testing a single substrate using the optimized concentration of relevant inhibitor: etomoxir (4 µM) to inhibit oxidation of LCFAs, UK5099 (2 µM) to inhibit the oxidation of glucose and/or pyruvate, and BPTES (3 µM) to inhibit oxidation of glutamine (final concentrations). These three assays are designed to be performed under conditions of saturating substrates with respect to glucose (10 mM), pyruvate (1 mM) and glutamine (2 mM) in the assay media. The source of long chain fatty acids is any endogenous stores of lipid/LCFAs in the cell used, and is cell-type dependent. **Figure 3** shows that the standard methods and assay conditions for each of the XF Substrate Oxidation Stress Tests are identical with the exception of the identity of the inhibitor used.

NOTE

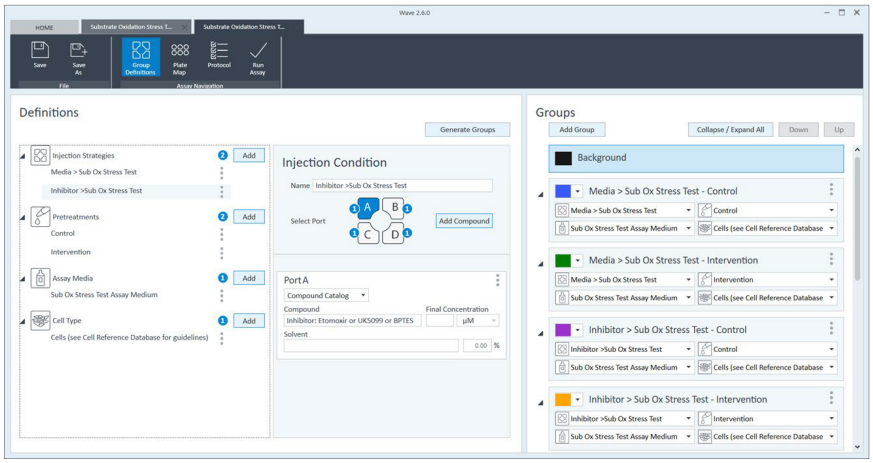
Users must first establish both the optimal cell seeding density and optimal FCCP concentration for ideal assay performance and resulting data. Typically, these are the same conditions established for a cell type in an XF Cell Mito Stress Test. For information on how to optimize cell seeding density and FCCP concentrations, please visit the Agilent Cell Analysis Learning Center website.

In these Standard Assays, decreased respiration rates in response to an inhibitor suggest that the cells have a demand or preference for that particular substrate under the experimental conditions established. In general, these assays can be used to facilitate investigation to address the following types of questions:

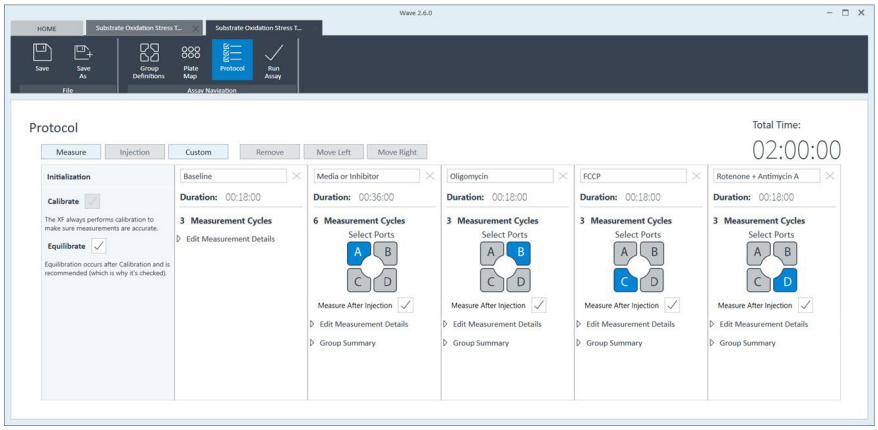
- Does the cell have a demand for a particular substrate or substrates?
- Is the cell highly reliant on a specific substrate, or can other substrates satisfy cellular demands?
- How is mitochondrial substrate demand and/or reliance affected if an intervention, such as genetic manipulation or drug exposure, is applied to the cell?

Like many XF assays, the Substrate Oxidation Stress Tests are typically performed subsequent to a pretreatment, or intervention, as designed by the researcher. This is shown as either a chronic intervention to the cells (for example, a genetic manipulation or long-term drug exposure), hours to days upstream of XF assay, or as an acute intervention (for example, drug exposure) just prior to the XF assay (**Figure 3**). In some cases, both chronic and acute interventions may be used (for example, rescue of genetic dysfunction through compound exposure). The kits may be used individually (such as focusing on one specific substrate) for investigating how a series of interventions or compounds may affect oxidation of that particular substrate; or in combination (such as focusing on two or more substrates) to elucidate overall effects of a given intervention with respect to substrate oxidation and mitochondrial function.

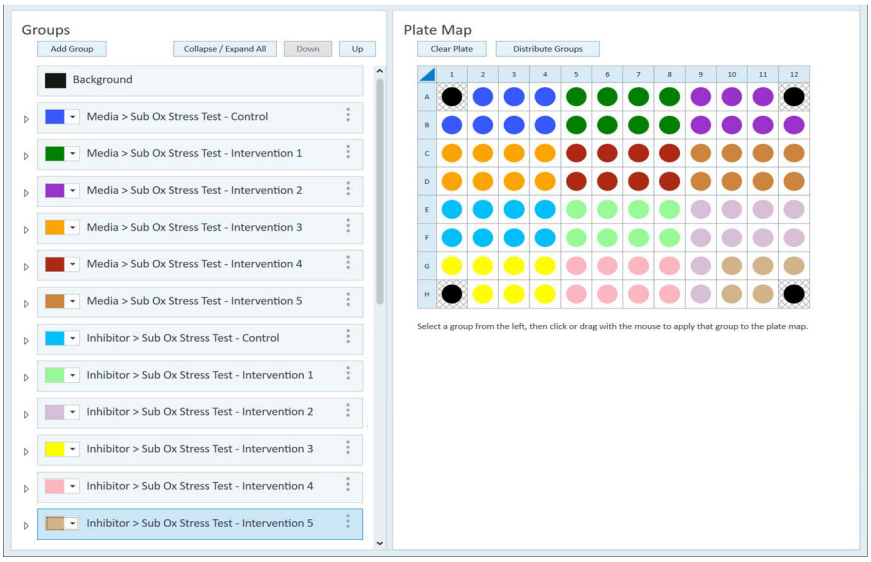
For examples of the design of assay templates for XF Substrate Oxidation Stress Tests - Standard Assays, refer to **Figure 4**.



4A. Starting group definitions for the Standard Substrate Oxidation Stress Test Assay Template. Here, the user must decide on the type of intervention(s) and the inhibitor identify used.

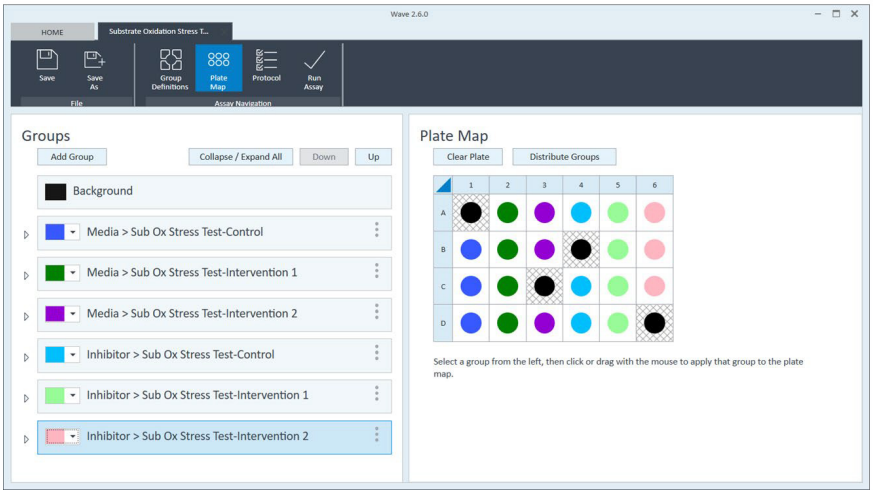


4B. Instrument protocol for XF/XFe96 analyzers. For XF/XFe24 analyzers, include the default 2-minute wait step required for 24-well XF platforms.

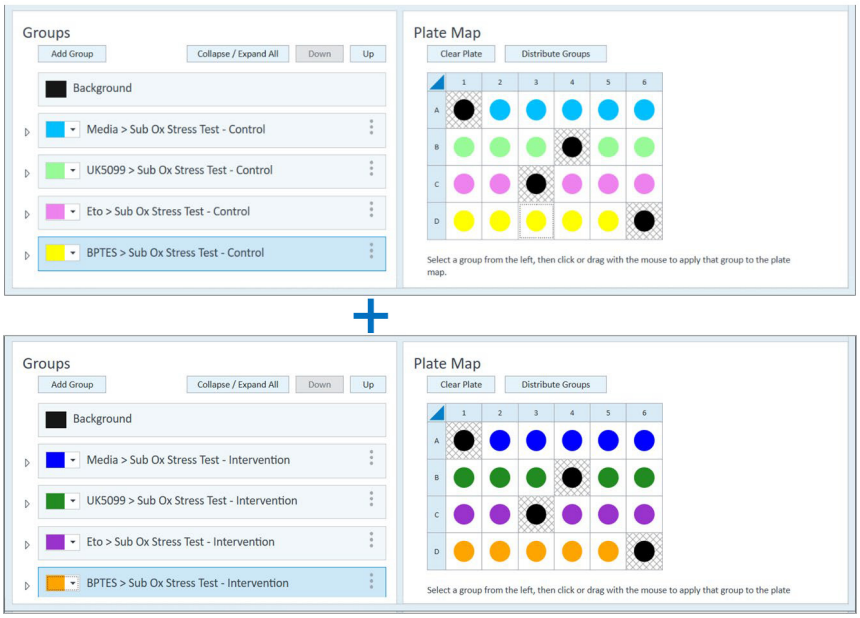


4C.
XFe96/XF96 plate map layout for testing the effects of multiple interventions (genetic variants and/or drug exposures) on the oxidation of a specific substrate (LCFAs or G/P or Q).

4D.
XFe96/XF96 plate map layout for testing the effects of a single intervention (genetic variant or drug exposure) on the oxidation of each substrate LCFAs and G/P and Q.



4E.
XFe24/XF24 plate map layout for testing the effects of multiple interventions (genetic variants and/or drug exposures) on the oxidation of a specific substrate (LCFAs or G/P or Q).



4F.

XFe24/XF24 plate map layout for testing the effects of a single intervention (genetic variant or drug treatment) on the oxidation of each substrate LCFAs and G/P and Q (Attention: 2 plates are required).

Figure 4. Agilent Seahorse XF Substrate Oxidation Stress Test - Assay Design Examples. These examples are suggested designs, and the user is encouraged to modify the generic Standard Substrate Oxidation Stress Test Assay Template file to accommodate the specific requirements of the experimental design.

XF Palmitate Oxidation Stress Test - Advanced Assay

Relevant kit includes:

XF Palmitate Oxidation Stress Test Kit (p/n 103693-100)

The XF Palmitate Oxidation Stress Test Advanced Assay details the workflow to specifically analyze the long chain fatty acid oxidation pathway in live cells. The kit includes the XF Palmitate-BSA FAO Substrate, L-carnitine, etomoxir, as well as oligomycin, FCCP, and rotenone/antimycin A, and is designed to determine the intrinsic rate and capacity of a cell to oxidize palmitate in the absence or limitation of other exogenous substrates. This workflow is best applied when investigating how interventions (genetic manipulations and drug exposure) specifically affect the LCFA oxidation process and can be a complementary and/or follow-up assay to the Standard Assay workflow for XF Long Chain Fatty Acid Oxidation Stress Test discussed in the previous section. The kinetic profile of the Advanced Palmitate oxidation assay and relevant assay parameters is outlined in **Figure 5**, and an overview of the experimental workflow for the Advance Assay is outlined in **Figure 6**.

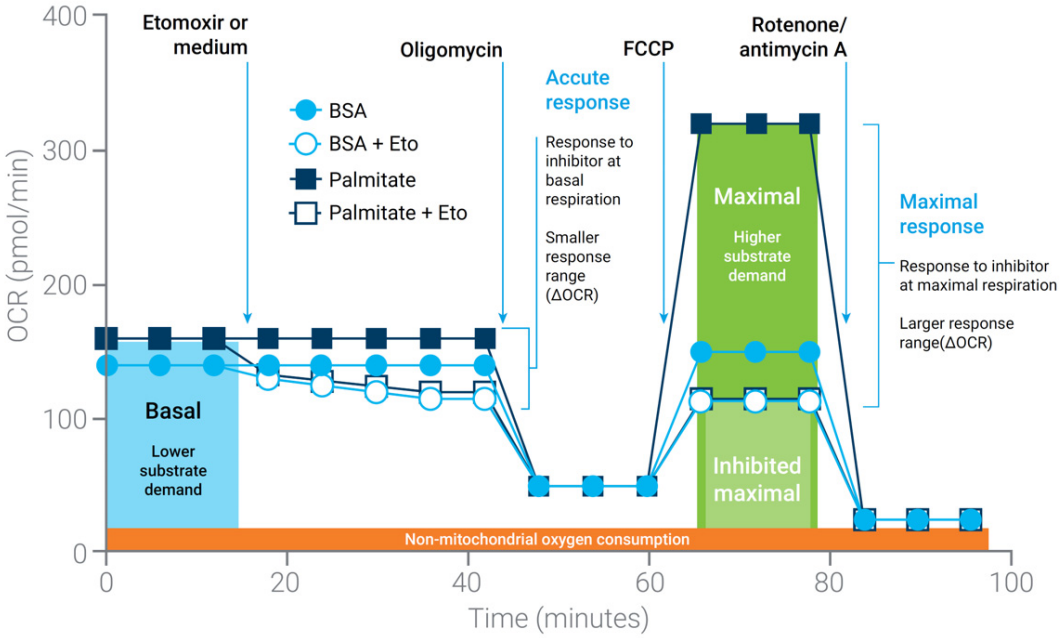


Figure 5. Agilent Seahorse XF Palmitate Oxidation Stress Test profile of the respiration parameters critical for palmitate demand. Sequential compound injections measure basal respiration, acute response to etomoxir, and maximal respiration in the absence and presence of etomoxir. Note that while minimal changes may be measured under basal conditions, such as the acute response, much larger responses are often revealed under conditions of high substrate demand (for example, FCCP), revealing differences in the ability of the cells to oxidize palmitate.

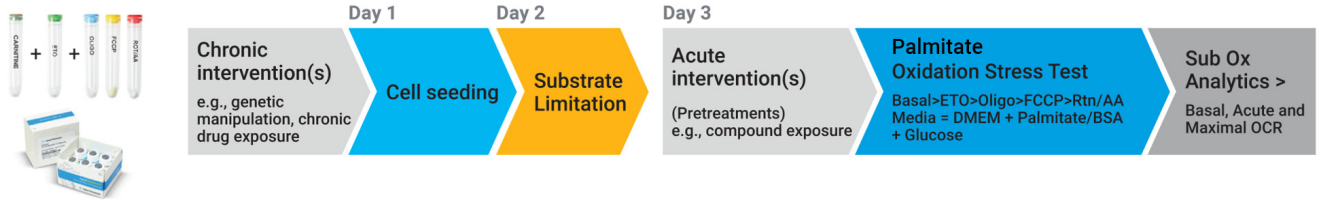


Figure 6. Agilent XF Palmitate Oxidation Stress Test - Advanced Assay. This assay is specifically focused on determining the intrinsic rate and capacity of a cell to oxidize palmitate in the absence or limitation of other exogenous substrates, using inhibitor etomoxir to inhibit oxidation of palmitate.

The Palmitate Oxidation Stress Test is designed to be performed under conditions of saturating palmitate (as palmitate-BSA) and L-carnitine, limited to no substrates provided with respect to glucose, pyruvate, and glutamine in the assay media. It is best applied when the experimental design calls for the cells to exclusively oxidize a long chain fatty acid, such as palmitate, to investigate effects of interventions specifically on the long chain fatty acid oxidation process.

As shown in **Figure 6**, this workflow often requires substrate limitation before and/or during the assay to condition the cells to obtain robust responses to the palmitate substrate added to the assay media. The suggested initial substrate-limited growth media conditions can be found in **Table 1**.

NOTE

Users must first establish the optimal cell density and optimal FCCP concentration for best assay performance and resulting data. Typically, these are the same conditions established for a cell type in an XF Cell Mito Stress Test, except the FCCP concentration may need to be re-optimized in the presence of BSA. For information on how to optimize cell density and FCCP concentrations, please visit the Agilent Cell Analysis Learning Center website.

Table 1 Suggested substrate limitation conditions for performing the XF Palmitate Oxidation Stress Test Advanced Assay with base growth media. Initial suggested time of incubation under substrate limitation is overnight (16 to 24 hours).

Base growth media	Growth media supplement	Suggested initial concentration in substrate-limited growth media
DMEM or RPMI without glucose, pyruvate, glutamine, or GlutaMAX	Glucose	0.5 mM
	Glutamine or GlutaMAX	1.0 mM
	Serum (for example, FBS)	1%
	Seahorse L-Carnitine	0.5 mM

Table 2 Suggested substrate limitation conditions for performing the XF Palmitate Oxidation Stress Test Advanced Assay with XF Assay Media.

XF Assay Media	Assay Media supplement		Suggested initial concentration in assay media
XF DMEM medium, pH 7.4 or XF RPMI medium, pH 7.4	XF Glucose		2.0 mM
	XF L-Carnitine		0.5 mM
	XF Palmitate-BSA*	96 well	30 µL/well
		24 well	85 µL/well
	XF BSA Control*	96 well	30 µL/well
		24 well	85 µL/well

* Note that XF Palmitate-BSA and XF BSA control are added directly to the XF cell culture plate wells just prior to starting the assay. See **pages 37 - 38** for further information.

NOTE **Optimal limited substrate concentrations and optimal time of incubation are cell-dependent and should be empirically determined for the cell type of interest.**

Instead of examination of substrate demand and reliance, this Advanced Assay is designed to be used when asking the following type of question:

- How an intervention, such as genetic manipulation or drug exposure, specifically affects the oxidation of long chain fatty acids when applied to the cell?

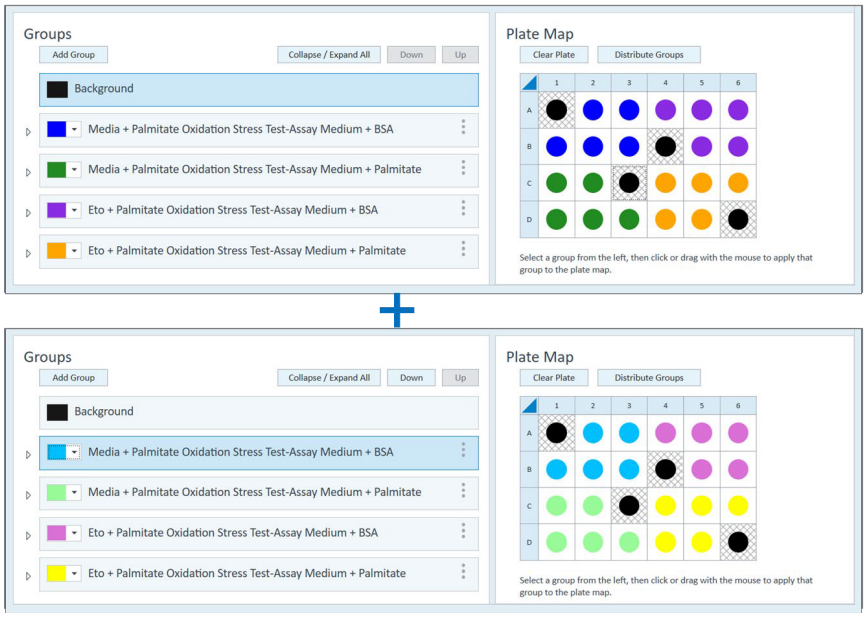
This assay is most often performed under some pretreatment condition, or intervention, as designed by the researcher. This is shown as either a chronic intervention to the cells (for example, a genetic manipulation or long-term drug exposure), hours to days upstream of XF assay, or as an acute intervention (for example, drug exposure) just prior to the XF assay (**Figure 6**). In some cases, both chronic and acute interventions may be used (for example, rescue of genetic dysfunction through compound treatment). For examples of the assay template design for XF Palmitate Oxidation Stress Test - Advanced Assay, refer to **Figure 7**.

There are critical and distinct difference between this Advanced Assay workflow and the Standard Assay workflow described in the previous section, including:

- Cells usually require a period of substrate limitation to measure oxidation of palmitate substrate provided, especially established cell lines. The specific conditions will be cell type dependent, and should be determined empirically for each cell type tested, particularly for primary type cells.
- An analogous BSA control group must be included for each condition tested to ensure that the observed responses are associated with Palmitate-BSA in the assay media. Therefore, the number of groups used doubles compared to a Standard Substrate Oxidation Stress Test. For examples of Palmitate Oxidation Stress Test assay templates, refer to **Figure 6**.
- L-carnitine is included in substrate-limited medium and assay medium to ensure that it is not a rate limiting factor in the assay.
- The conditions for substrate limitation and performance of the Advanced Assay are only validated for use with the XF Palmitate-BSA substrate and BSA control.

7A.

XFe96/XF96 plate map layout for testing the effects of a single intervention (genetic variant or drug exposure) specifically on the oxidation of palmitate by the cell.



7B.

XFe24/XF24 plate map layouts for testing the effects of a single intervention (genetic variant or drug exposure) specifically on the oxidation of palmitate by the cell. Attention: 2 plates are required.

Figure 7. Agilent XF Palmitate Oxidation Stress Test - Plate Layout Map Examples.

Glossary

- **Basal Respiration:** Oxygen consumption used to meet cellular ATP demand and resulting from mitochondrial proton leak. Shows substrate demand of the cell under baseline conditions.
- **Acute Response:** Change in oxygen consumption rate due to an injection of substrate oxidation inhibitor (etomoxir, UK5099, BPTES). Reported as a change in OCR (i.e., ΔOCR pmol/min).
- **Maximal Respiration:** The maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP increases a substrate demand by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, and amino acids) to meet this metabolic challenge. Shows substrate demand of the cell under maximal respiration conditions.
- **Non-mitochondrial Respiration:** Oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after rotenone/antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration.
- **Standard Substrate Oxidation Stress Test Workflow:** Three complementary assays, each focused on testing cellular demand of a single substrate (LCFAs or G/P or Q) using the optimized concentration of relevant inhibitor: etomoxir or UK5099 or BPTES. Designed to be performed under conditions of saturating substrates with respect to glucose, pyruvate, and glutamine in the assay media (Figures **2**, **3**, **4**, and **8**).
- **Advanced XF Palmitate Oxidation Stress Test:** A single assay designed to determine the intrinsic rate and capacity of a cell to oxidize palmitate in the absence or limitation of other exogenous substrates. Performed under conditions of saturating palmitate (as palmitate-BSA) and L-carnitine, in the assay media. It is best applied when the experimental design calls for the cells to exclusively oxidize a long-chain fatty acid, such as palmitate, in order to investigate effects of interventions specifically on the long chain fatty acid oxidation process (Figures **5**, **6**, **7**, **12**, and **Table 1**).
- **MST:** XF Cell Mito Stress Test, a well-recognized assay that provides a comprehensive view of mitochondrial function by reporting multiple parameters for mitochondrial respiration. For more information, visit the **Agilent Seahorse XF Cell Mito Stress Test** product website.
- **FAO:** Fatty acid oxidation.
- **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. Note that concentrations in excess of 4 µM (final in assay) result in substantial off-target effects on mitochondrial respiration
- **UK5099:** An inhibitor of the glucose oxidation pathway. UK5099 inhibits the mitochondrial pyruvate carrier (MPC), which transports pyruvate into the mitochondria. 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. Note that BPTES does not inhibit GLS2.
- **LCFAs:** Long chain fatty acids.
- **G/P:** Glucose/pyruvate.
- **Q:** Glutamine.

- **L-Carnitine:** A supplement provided in the Palmitate Oxidation Stress Kit for use in substrate limited growth media and substrate limited assay media. Ensures concentrations of L-carnitine are saturating and not limiting rates of palmitate oxidation.
- **Growth Media:** Fully supplemented cell culture media appropriate for the specific cell type.
- **Substrate Limitation:** A condition used in the Palmitate Oxidation Stress Test - Advanced Assay. It usually refers to a period of time (e.g., overnight) in which key substrates (typically glucose, pyruvate, GlutaMAX, and serum) are reduced in concentration in the cell culture media.
- **Substrate-limited growth media:** Cell culture media with key substrates (typically glucose, pyruvate, GlutaMAX, and serum) reduced in concentration (see **Table 1**).
- **Substrate-limited assay media:** Assay media with key substrates (typically glucose, pyruvate, and glutamine) reduced in concentration and/or omitted (see **Table 2**).

References

1 Divakaruni, A. S.; *et al.* **2018**. 'Etomoxir Inhibits Macrophage Polarization by Disrupting CoA Homeostasis', Cell Metab, 28: 490-503.e7.

2 Divakaruni, A. S.; *et al.* **2014**. 'Analysis and interpretation of microplate-based oxygen consumption and pH data', Methods Enzymol, 547: 309-54.

3 Hildyard, J. C.; *et al.* **2005**. 'Identification and characterisation of a new class of highly specific and potent inhibitors of the mitochondrial pyruvate carrier', Biochim Biophys Acta, 1707: 221-30.

4 Robinson, Mary M.; *et al.* **2007**. 'Novel mechanism of inhibition of rat kidney-type glutaminase by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES)', Biochemical Journal, 406: 407-14.

2 Kit Information

Kit Contents

Products relevant to the Seahorse XF Substrate Oxidation Stress Tests include four Kits. Each kit contains reagents sufficient for three complete XF Substrate Oxidation Stress Tests in either a 96- or 24-well Agilent Seahorse XF Cell Culture Microplate. The contents or components for each kit are different, depending on the substrate being examined, and are listed in **Table 3**.

Table 3 Agilent Seahorse XF Substrate Oxidation Stress Test Kits contents are shown per pouch, three (3) pouches or vials (Palmitate and BSA control) per kit.

Compound	Cap color	Qty per vial
Agilent Seahorse XF Long Chain Fatty Acid Oxidation Stress Test kit contents (p/n 103672-100)		
Etomoxir	Green	112 nmol
Oligomycin	Blue	63 nmol
FCCP	Yellow	72 nmol
Rotenone/Antimycin A	Red	27 nmol each
Agilent Seahorse XF Glucose/Pyruvate Oxidation Stress Test kit contents (p/n 103673-100)		
UK5009	Orange	56 nmol
Oligomycin	Blue	63 nmol
FCCP	Yellow	72 nmol
Rotenone/Antimycin A	Red	27 nmol each
Agilent Seahorse XF Glutamine Oxidation Stress Test kit contents (p/n 103674-100)		
BPTES	Grey	84 nmol
Oligomycin	Blue	63 nmol
FCCP	Yellow	72 nmol
Rotenone/Antimycin A	Red	27 nmol each
Agilent Seahorse XF Palmitate Oxidation Stress Test kit contents (p/n 103693-100)		
Etomoxir	Green	112 nmol
Oligomycin	Blue	63 nmol
FCCP	Yellow	72 nmol
Rotenone/Antimycin A	Red	27 nmol each
XF Palmitate-BSA	-	2 mL
XF BSA control	-	2 mL
Seahorse L-carnitine	Brown	10 mg

Kit Shipping and Storage

Products are shipped at ambient temperature, and can be stored at room temperature, except for XF Palmitate BSA FAO substrate. The XF Palmitate BSA FAO substrate is shipped with ice packs, and should be stored at -20 °C upon arrival. All kits are stable for one year from the date of manufacture, while XF L-carnitine is stable for two years from the date of manufacture. The kit expiration date is printed on the label of the kit box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 3 and 12 months.

Additional Agilent Products Required

The following products are also required for performing the Seahorse XF Substrate Oxidation Stress Tests but not supplied with the kits. For a complete list of materials required to perform an XF assay, please visit the **Agilent Cell Analysis Assay Learning Center** website.

Table 4 Additional required items.

Item	Vendor	Part number
Seahorse XF96, XF24, XFe96 or XFe24 Analyzer	Agilent Technologies	
Seahorse XF DMEM Medium, pH 7.4* or	Agilent Technologies	103575-100
Seahorse XF RPMI Medium, pH 7.4*	Agilent Technologies	103576-100
Seahorse XF FluxPak for the Analyzer being used	Agilent Technologies	Various
Seahorse XF 1.0 M Glucose solution	Agilent Technologies	103577-100
Seahorse XF 100 mM Pyruvate solution†	Agilent Technologies	103578-100
Seahorse XF 200 mM Glutamine solution†	Agilent Technologies	103579-100

* XF DMEM or RPMI media can also be purchased together with the supplements listed in this table as bundled products (p/n 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**.

† Not required when performing the Seahorse XF Palmitate Oxidation Stress Test kit (p/n 103693-100).

3 Assay Workflow for Standard Substrate Oxidation Stress Test

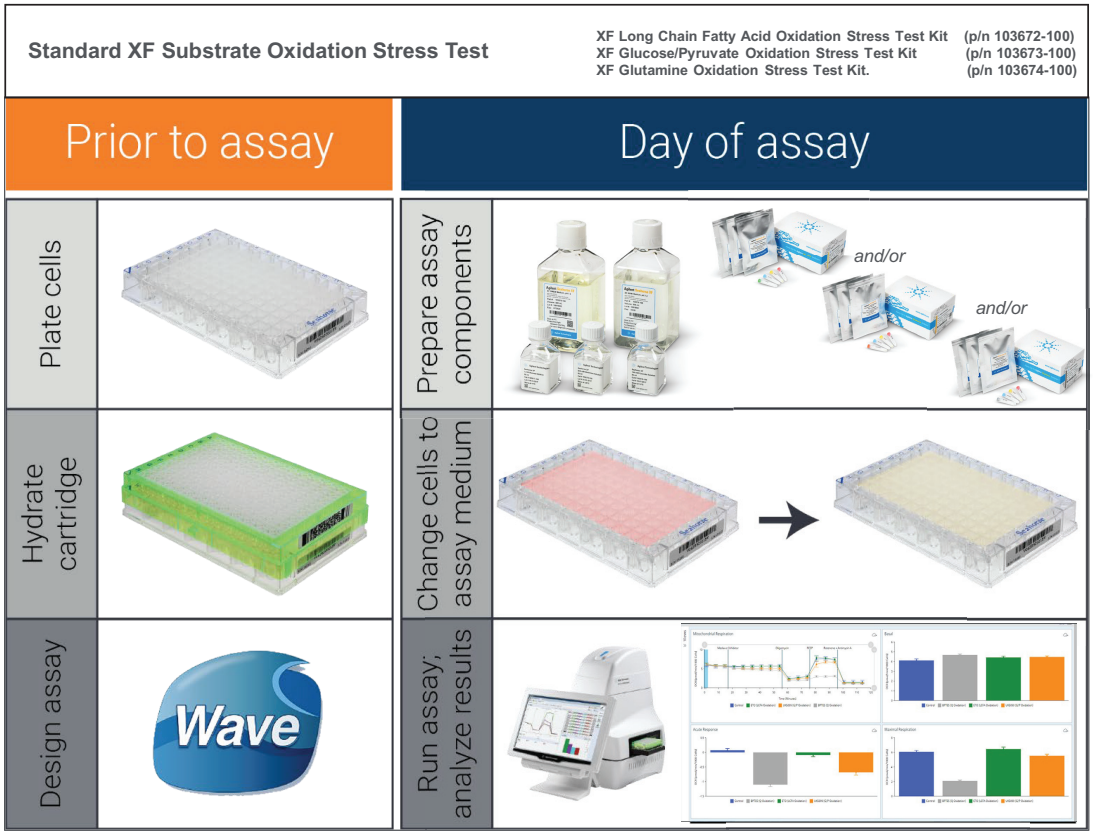


Figure 8. Agilent Seahorse XF Substrate Oxidation Stress Test-Standard Assay Workflow.

NOTE

Optimal cell seeding density and FCCP concentration should be empirically determined for your cell type prior to the assay. For more details, please refer to the Basic Procedures on Agilent Cell Analysis Learning Center website.

The Cell Line Reference Database is an excellent resource for finding information regarding the cell type of interest.

One Day Prior to Assay

1 Turn on the Seahorse XFe/XF96 or XFe/XF24 Analyzer, and let it warm up overnight to allow the temperature to stabilize (minimum five hours).

2 Hydrate a sensor cartridge in sterile or distilled water at 37 °C in a non-CO_2 incubator overnight. For more information, refer to the Basic Procedure, "Hydrating the sensor cartridge", on the **Agilent Cell Analysis Learning Center** website.

3 For adherent cells, plate cells at a pre-determined density in the Seahorse XF Microplate using the appropriate cell culture growth medium. Refer to the **Agilent Cell Analysis Learning Center** website for details.

For suspension cells, see section: Day of Assay/Prepare Seahorse XF Cell Culture Microplate for Assay.

4 Create an Assay Template file with Wave Desktop software using the Standard Substrate Oxidation Stress Test Assay Template. Make any necessary group modifications to the template for your specific assay design.

Day of Assay

Complete hydration of sensor cartridge by removing water, adding the appropriate volume of XF calibrant to each well, and placing in a 37 °C non-CO_2 incubator for 60 minutes.

Prepare assay media

1 Prepare Standard Substrate Oxidation Stress Test assay media by supplementing 97 mL of Seahorse XF DMEM Medium, pH 7.4 with 1.0 mL each of XF glucose, XF pyruvate, and XF glutamine (10 mM, 1 mM, and 2 mM final in assay media, respectively). These are the recommended initial conditions. However, the desired assay medium composition can be modified if needed.

Table 5 Standard Substrate Oxidation Stress Test Assay Media.

Component	Volume	Final concentration
Agilent Seahorse XF DMEM or RPMI Medium, pH 7.4	97 mL	-
XF 1.0 M Glucose Solution	1.0 mL	10 mM
XF 100 mM Pyruvate Solution	1.0 mL	1 mM
XF 200 mM Glutamine Solution	1.0 mL	2 mM

2 Warm media to 37 °C until it is ready to use.

NOTE **No pH adjustment to the assay media is necessary when recommended supplement concentrations are used.**

Prepare Seahorse XF Cell Culture Microplate for assay

For adherent cells

1. Remove cell culture microplate from 37 °C cell culture incubator and examine cells under microscope to confirm consistent plating and proper cell morphology.
2. Remove the growth medium from the cell culture microplate. Wash once with warmed assay medium. Add assay medium to a final volume of 180 µL/well for 96-well plates and 500 µL/well for 24-well plates. Refer to the **Agilent Cell Analysis Learning Center** for more details.
3. Incubate cell plates with assay medium at 37 °C in a non-CO_2 incubator for 45 to 60 minutes prior to the assay.
4. Before starting the XF assay, remove the assay medium AGAIN and add fresh, warm assay medium to each well. Total volume should be 180 µL/well for 96-well plates and 500 µL/well for 24-well plates.

For suspension cells

1. Pellet cells out of their growth medium and resuspend in warm assay medium.
2. Count cells and suspend at a concentration such that seeding 50 µL (XF96/XFe96) or 100 µL (XFe24) of cells contains the desired cell number per well, leaving four wells without cells as background correction wells.
3. Add desired cells/well, then centrifuge gently to adhere.
4. Gently add assay medium to each well. Total well volume should be 180 µL/well for 96-well plates and 500 µL/well for 24-well plates.
5. Incubate the plate at 37 °C in a non-CO_2 incubator for 45 to 60 minutes prior to the assay.

Refer to the **Agilent Cell Analysis Learning Center** for more information on preparation of suspension cells for XF assays.

Prepare compound stock solutions and working solutions

CAUTION **Use compounds on the same day they are reconstituted. Do not freeze and reuse. Discard any remaining compound solutions.**

1. Remove one foil pouch and the decapper from the kit box.
2. Open pouch and remove all four vials: Oligomycin (blue cap), FCCP (yellow cap), Rotenone/Antimycin A (red cap), and the inhibitor vial (specific to each kit; either etomoxir (green cap), UK5099 (orange cap), or BPTES (grey cap)).
3. Tap down the vials to ensure powder is on the bottom of the vial before removing the vial cap using the decapper provided with the kits.

4 Resuspend the content in each vial with the appropriate volume of prepared assay medium as described in **Table 6**. Vortex ~1 minute to ensure full resuspension of compounds. These are compound stock solutions.

Table 6 Stock solutions.

Compound	Volume of assay medium	Stock concentration
Etomoxir from XF Long Chain Fatty Acid Oxidation Stress Test kit or	700 µL	160 µM
UK5099 from XF Glucose/Pyruvate Oxidation Stress Test kit or	700 µL	80 µM
BPTES from XF Glutamine Oxidation Stress Test kit	700 µL	120 µM
Oligomycin	420 µL	150 µM
FCCP	720 µL	100 µM
Rotenone/Antimycin A	540 µL	50 µM

5 Using the compound stock solutions to prepare working solutions for loading into the injection ports on sensor cartridges.

6 Prepare 2 or 3 mL working solutions for each compound in assay medium as indicated in **Table 7** for XF/XFe96 sensor cartridges and **Table 8** for XF/XFe24 sensor cartridges. It is recommended to use 1.5 µM of oligomycin and 0.5 µM rotenone/antimycin A (final concentration) for most cells. Optimal FCCP concentration should be determined prior to the assay.

Table 7 Compound preparation for loading to XFe/XF96 sensor cartridges. Starting assay medium volume for cell plate is 180 µL per well.

	Final well µM	Stock solution volume (µL)	Medium volume (µL)	10× (Port) µM	Volume added to port (µL)
Port A: Etomoxir or	4	500	1,500	40	20
UK5009 or	2	500	1,500	20	20
BPTES	3	500	1,500	30	20
Port B: Oligomycin	1.5	300	2,700	15	22
Port C: FCCP	0.25	75	2,925	2.5	25
	0.5	150	2,850	5	25
	1.0	300	2,700	10	25
	2.0	600	2,400	20	25
Port D: Rotenone/Antimycin A	0.5	300	2,700	5	27

Table 8 Compound preparation for loading to XFe/XF24 sensor cartridges. Starting assay medium volume for cell plate is 500 µL per well.

	Final well µM	Stock solution volume (µL)	Medium volume (µL)	10× (Port) µM	Volume added to port (µL)
Port A: Etomoxir or	4	500	1,500	40	56
UK5009 or	2	500	1,500	20	56
BPTES	3	500	1,500	30	56
Port B: Oligomycin	1.5	300	2,700	15	62
Port C: FCCP	0.25	75	2,925	2.5	69
	0.5	150	2,850	5	69
	1.0	300	2,700	10	69
	2.0	600	2,400	20	69
Port D: Rotenone/Antimycin A	0.5	300	2,700	5	75

Load the injection ports on sensor cartridge

Proper port loading techniques can be found in Basic Procedure "Loading the Sensor Cartridge with Compounds", on the **Agilent Cell Analysis Learning Center** website.

Please read the information prior to loading compounds. Ensure that the sensor cartridge is properly hydrated prior to use.

For the location of the ports, please refer to **Figure 9**.

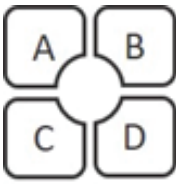


Figure 9. Layout of injection ports on sensor cartridges for XFe96, XF96, and XFe24 sensor cartridges. Note the XF24 sensor cartridges have a different layout. Please consult the XF Assay Learning Center for XF24 injection port layout.

Refer to **Table 9** for loading volume and port designation for compounds in different types of assays.

Table 9 Recommended injection volumes for Substrate Oxidation Stress Test injection schemes. Starting assay medium volume is 180 µL per well for 96-well cell plates and 500 µL per well for 24-well cell plates.

			Add to port volume	
Port	Compound	Port concentration	96 well	24 well
A	Inhibitor (etomoxir, UK5099, or BPTES)*	10×	20 µL	56 µL
B	Oligomycin	10×	22 µL	62 µL
C	FCCP	10×	25 µL	69 µL
D	Rotenone/Antimycin A	10×	27 µL	75 µL

* For controls, assay medium should be used in port A instead of inhibitors.

Load template onto the Seahorse XFe Analyzer

NOTE **If templates(s) are already present on the XFe analyzer, skip this step.**

Personal computer (internet access required)

1 Use the hyperlink below to download the **Seahorse XF Substrate Oxidation Stress Test - Standard Template** (for Long Chain Fatty Acid, and/or Glucose/Pyruvate, and/or Glutamine Oxidation Stress Tests).
www.agilent.com/cs/library/software/public/software-agilent-seahorse-xf-subox-assay-template-files-cell-analysis-agilent.zip

2 Unzip the folder, then copy and paste the assay template files to a USB drive or network drive (if Seahorse XFe Analyzer is networked).

Seahorse XFe96/XFe24 Analyzer

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

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

3 Locate the Assay Template file to import on the USB or network drive.

4 Click **Open** in the Windows dialogue box. The imported Assay template will be available for selection from the Templates view on the XFe Analyzer.

5 Repeat for next template, if applicable.

6 The imported Assay template(s) will now be available for selection in the list of available templates.

Running the XF substrate oxidation stress test assay

1 Select the **Seahorse XF Substrate Oxidation Stress Test-Standard** template from the list of available templates and click **Open File** (or double-click the template).

2 Group Definitions: confirm or modify the default groups and conditions for your assay.

3 Plate Map: confirm or modify the plate layout map for your assay.

4 Protocol: No action required - confirm or modify the Instrument Protocol for additional measurements cycles during the assay.

5 Run Assay: Click **Start Run** when ready.

6 When prompted, remove the cartridge lid and place the loaded sensor cartridge with the utility plate on the thermal tray of the Seahorse XFe Analyzer. Ensure correct plate orientation and the cartridge lid has been removed. Then, click **I'm Ready**. Calibration takes approximately 15 to 30 minutes.

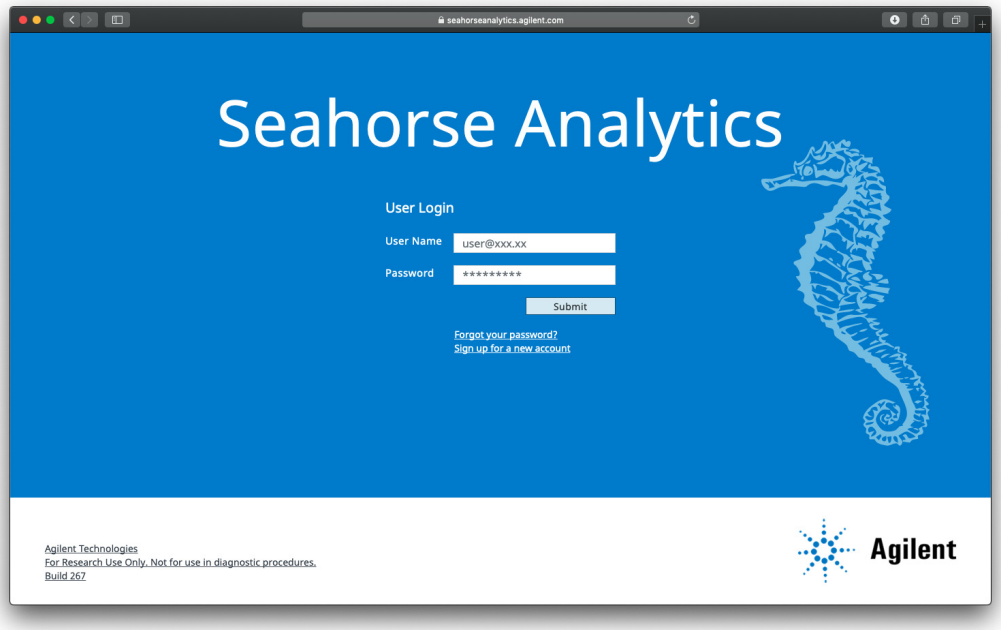
7 After completing Calibration, Wave Controller will display the Load Cell Plate dialog. Click **Open Tray** to eject Utility Plate and load the Cell Plate. Ensure the lid is removed from Cell Plate before Loading.

8 Click **Load Cell Plate** to run the assay.

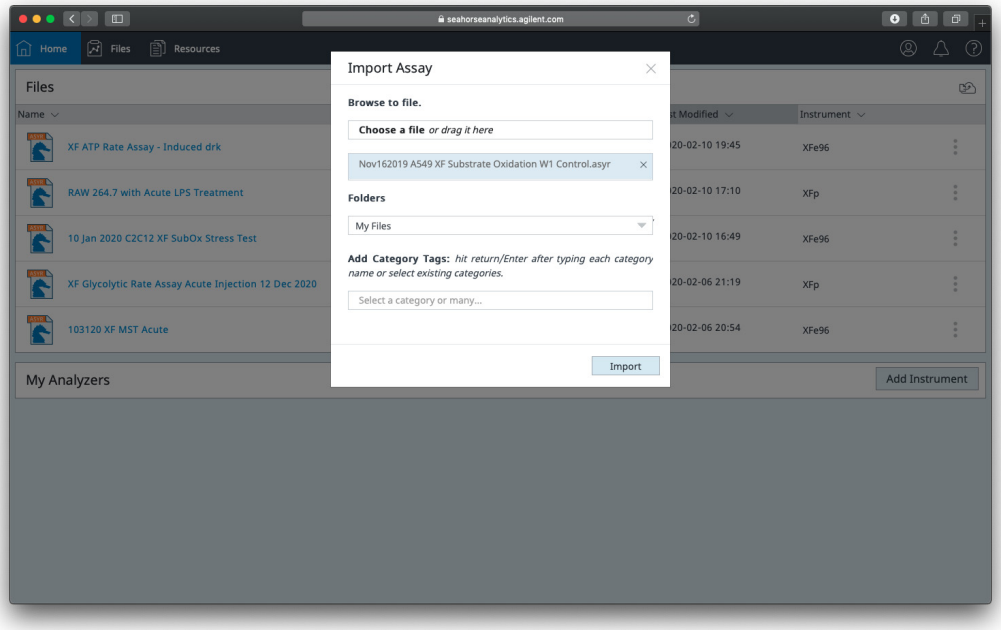
Data Analysis Using Agilent Seahorse Analytics

Agilent Seahorse Analytics is a new, web-based software platform that provides a simple, streamlined data analysis workflow for the XF Substrate Oxidation Stress Test assay. Seahorse Analytics automatically calculates the Substrate Oxidation Stress Test Parameters: basal respiration, acute response to inhibitor, and maximal respiration. Visit **https://seahorseanalytics.agilent.com** to register or log in to your Seahorse Analytics account.

1. After the assay is completed, transfer your assay result file to your personal computer using a USB drive or network drive.
2. Go to **https://seahorseanalytics.agilent.com** to register or log in to your Seahorse Analytics account.

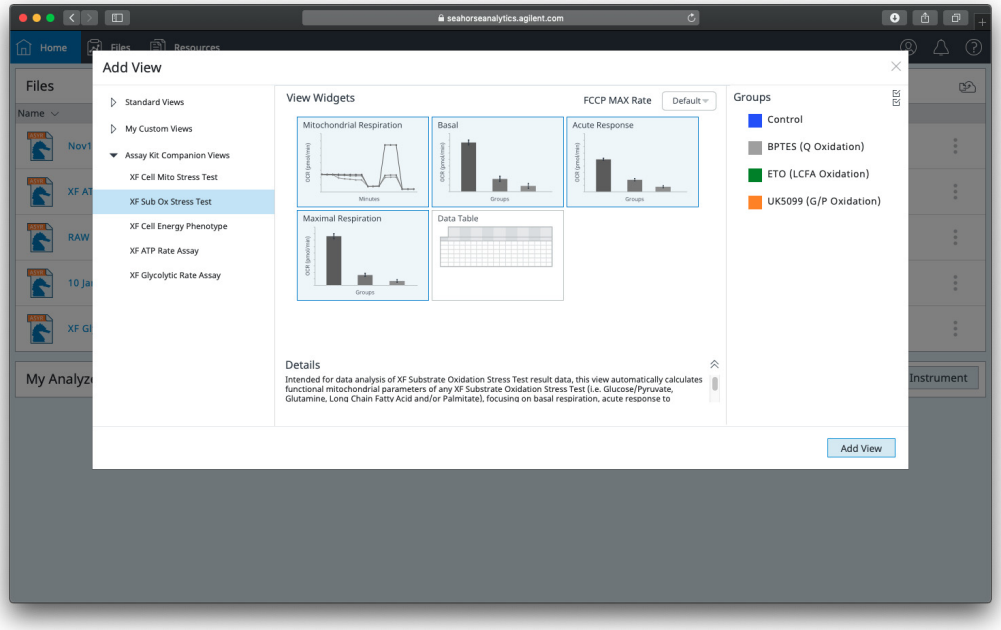


3 Import the assay result file to your account.



4 Open the assay result file, and select the XF Substrate Oxidation Stress Test analysis view found under the Assay Kit Companion views menu.

5 Select groups to add to the analysis view, then click **Add View**.



Examples of Data

Figure 10 shows example Standard Substrate Oxidation Assay data from Seahorse Analytics for A549 cells.

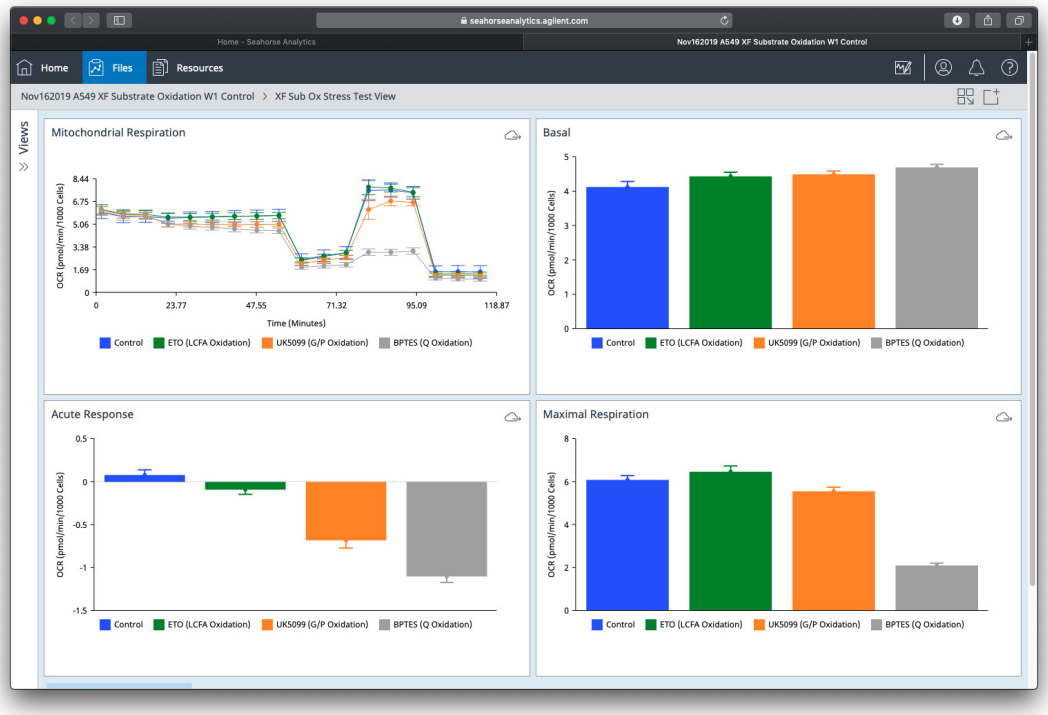


Figure 10. Standard Substrate Oxidation Assay data derived from Agilent Seahorse Analytics for A549 cells.

Figure 11 shows example data from experiments with A549 (Panel A), C2C12 (Panel B), and HepG2 cells (Panel C).

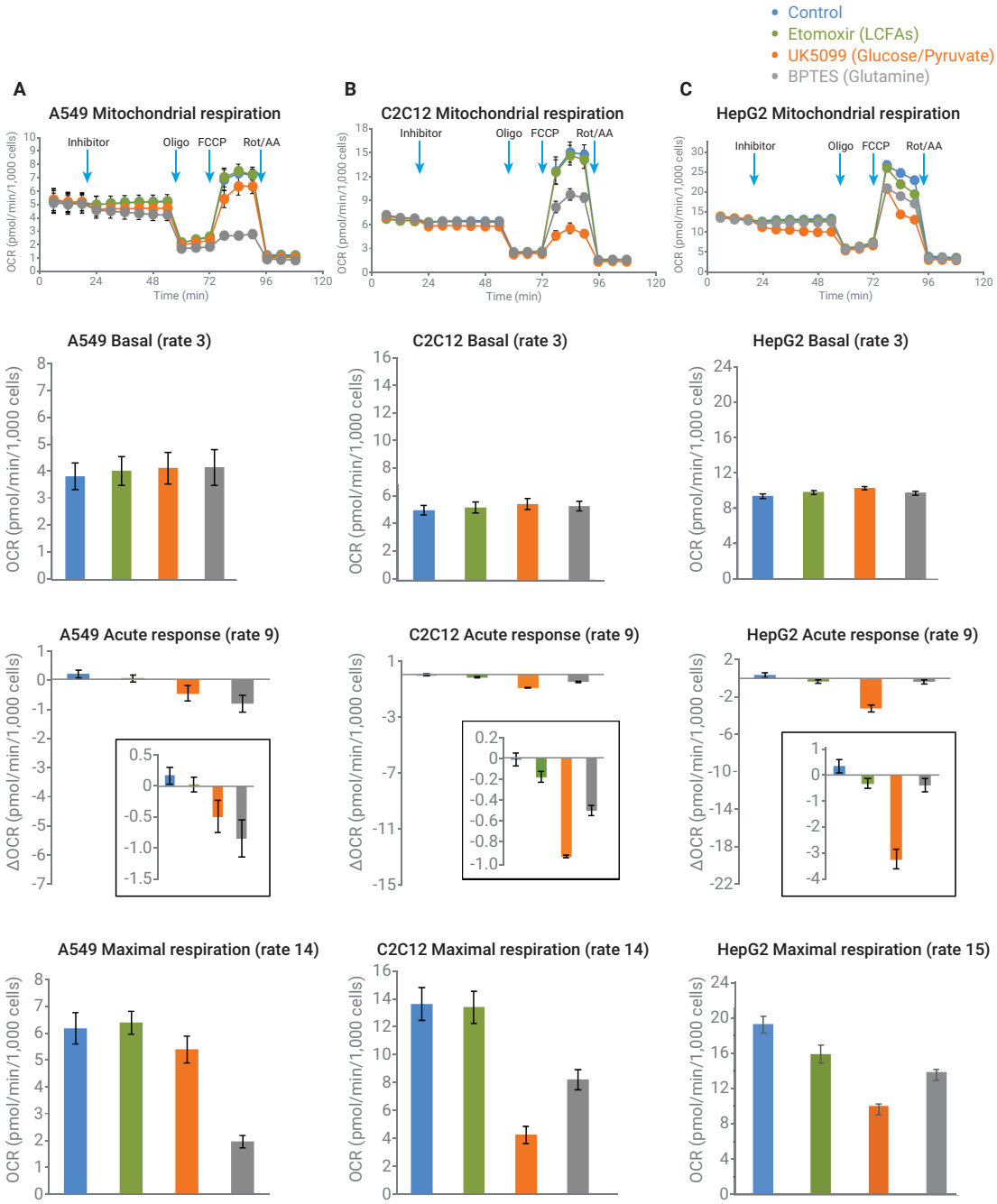


Figure 11. Comparing substrate oxidation in A549, C2C12 and HepG2 cells using XF Glucose Pyruvate, Long Chain Fatty Acid, and Glutamine Oxidation Stress Tests. A549 (A), C2C12 (B), and HepG2 (C) cells were seeded in XF96 Cell Culture Plates and grown overnight. Cells were subject to XF Substrates Oxidation Stress Tests using injections of assay media (control), etomoxir (LCFA oxidation), UK5099 (glucose/pyruvate oxidation) or BPTES (Glutamine oxidation), followed by common sequential injections of oligomycin, FCCP, and Rtn/AA. XF Assay Media = XF DMEM, pH 7.4 supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine. Each cell type was assayed on three individual days, with result data being compiled and processed through Seahorse Analytics. Error is reported as ± SEM. Similar results for all cell types tested were obtained using an XFe24 Analyzer (data not shown).

Inspection of the Basal Respiration parameter for each cell type reveals the expected results with all conditions showing identical basal OCRs before inhibitors are added. Upon injection of inhibitor, each cell type displays a detectable, but relatively small, acute response with respect to respiration under basal assay conditions. However, under conditions of maximal respiration (such as under conditions of higher substrate demand by the mitochondria), responses to inhibitors are significantly increased, and different responses to different inhibitors become apparent across the different cell types tested. Please see the Agilent application note: Revealing Cellular Metabolic Phenotype and Function Using Agilent XF Substrate Oxidation Stress Tests for further discussion regarding interpretation of substrate demand for each cell type, as well as suggestions for Standard Substrate Oxidation Stress Test experimental design in the context of cancer cell and immune cell biology and drug discovery.

4 Assay Workflow for Palmitate Oxidation Stress Test - Advanced Assay

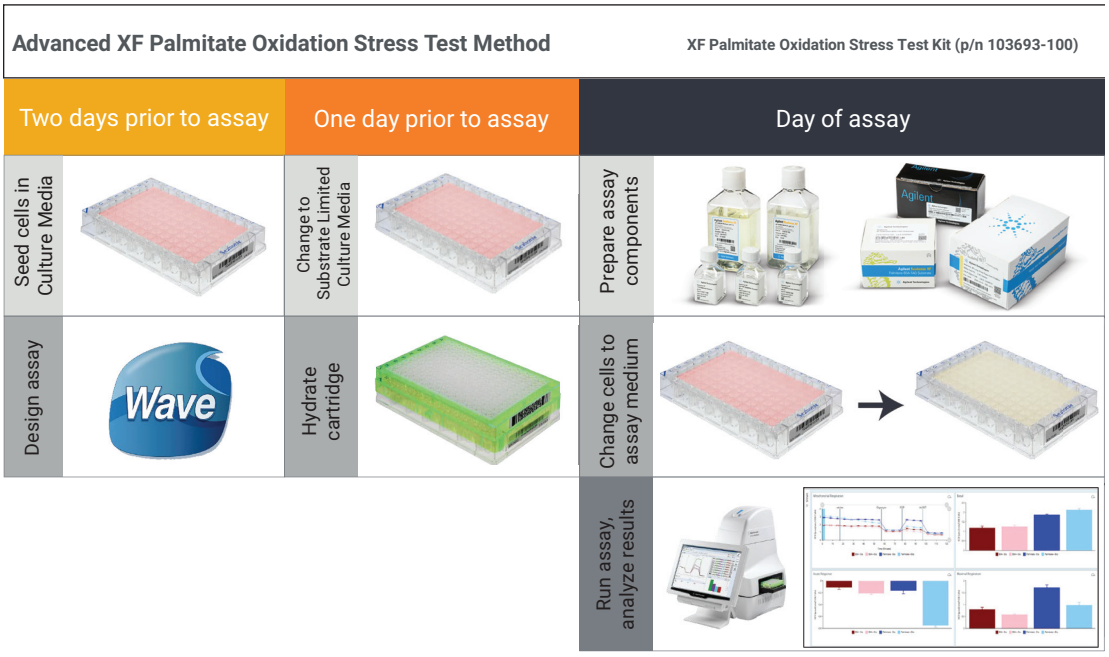


Figure 12. Agilent Seahorse XF Palmitate Oxidation Stress Test Workflow - Advanced Assay workflow.

Two Days Prior to Assay

For adherent cells, plate cells at a predetermined density in the Seahorse XF Tissue Culture plate using the appropriate cell culture growth medium. Refer to the **Agilent Cell Analysis Learning Center** website for details.

One Day Prior to Assay

Prepare substrate-limited growth media

1. Remove one foil pouch from Agilent Seahorse L-Carnitine box. Open pouch and remove the L-carnitine vial. Bring to a cell culture hood.
2. Add 100 µL sterile, tissue culture-grade water to the L-carnitine vial. The resulting stock concentration is 0.5 M.
3. Aliquot 18.5 mL unsupplemented growth media (without glucose, pyruvate, glutamine, or GlutaMAX) to a sterile 50 mL conical tube in a cell culture hood.
4. Supplement with 0.5 mM glucose, 1 mM glutamine or GlutaMAX, and 1% fetal bovine serum (final concentrations). Also add 20 µL of carnitine prepared in Step 2. This is the substrate-limited growth media with a final carnitine concentration of 0.5 mM.
5. Place the remainder of carnitine stock solution at 4 °C after use. This carnitine solution will also be used to supplement the assay media the next day.

Prepare cell plates

1. For both adherent cells or suspension cells, replace the cell growth media with substrate-limited growth media. It is recommended to wash the cell plate one time with either PBS or substrate-limited growth media to ensure the complete removal of residual growth media.
2. Add 100 µL of substrate-limited growth media in each well for 96-well plates and 250 µL in each well for 24-well plates.
3. Place the cell plates back to 37 °C cell culture incubator.

Other preparation

1. Turn on the Seahorse XFe/XF96 or XFe/XF24 Analyzer and let it warm up overnight to allow the temperature to stabilize (minimum five hours).
2. Hydrate a sensor cartridge in sterile or distilled water at 37 °C in a non-CO_2 incubator overnight. For more information, refer to the Basic Procedure, “Hydrating the sensor cartridge”, on the **Agilent Cell Analysis Learning Center** website.
3. Create an Assay Template file with Wave Desktop software using the Palmitate Oxidation Stress Test Advanced Assay Template. Make any necessary group modifications to the template for your specific assay design.

Day of Assay

Complete Hydration of sensor cartridge: remove water and add the appropriate volume of XF calibrant to each well and replace in a 37 °C non-CO_2 incubator for 60 minutes.

Prepare assay media

1 Prepare Advanced Palmitate Oxidation Stress Test assay media by supplementing 75 mL of Seahorse XF DMEM or RPMI Medium, pH 7.4 with 2 mM of XF glucose and 0.5 mM L-carnitine (no pyruvate or glutamine). This is the recommended initial condition for glucose concentration and can be modified if needed.

Table 10 Advanced Palmitate Oxidation Stress Test Assay Media.

Component	Volume	Final concentration
Agilent Seahorse XF DMEM or RPMI Medium, pH 7.4	75 mL	-
XF Glucose	150 µL	2.0 mM
Seahorse L-Carnitine	75 µL	0.5 mM

2 Warm up at 37 °C until ready to use. (Palmitate-BSA substrate will be added immediately before the assay.)

NOTE

No pH-adjustment to the assay medium is necessary when recommended supplement concentrations are used. Also, remove Palmitate-BSA and BSA control from the freezer. Warm to 37 °C before use.

Prepare Seahorse XF Cell Culture Microplate for assay

For Adherent Cells

1 Remove cell culture microplate from 37 °C CO_2 incubator and examine cells under microscope to confirm consistent plating and proper cell morphology.

2 Remove the substrate-limited growth medium from the cell culture microplate. Wash once with warmed assay medium using. Add assay medium to a final volume of 180 µL/well for 96-well plates and 500 µL/well for 24-well plates. Refer to the **Agilent Cell Analysis Learning Center** for more details.

3 Incubate cell plates with substrate limited assay medium at 37 °C in a non-CO_2 incubator for 45 to 60 minutes prior to the assay.

4 Before starting the XF assay, remove the assay medium from cell plates again and add fresh, warm assay medium to each well to a volume of 150 µL for 96-well plates and 415 µL for 24-well plates.

5 Finally, just prior to starting the assay, add 30 µL of 1X Palmitate-BSA or 30 µL of 1X BSA control to the appropriate wells in 96-well plates. Or add 85 µL of 1X Palmitate-BSA or 85 µL of 1X BSA control to the appropriate wells in 24-well plates. The total volumes before the assay are 180 µL/well for 96-well plates and 500 µL/well for 24-well plates.

For Suspension Cells

1 Pellet cells out of their growth medium and resuspend in warm assay medium.

2 Count cells and suspend at a concentration such that seeding 50 µL (XF96/XFe96) or 100 µL (XFe24) of cells contains the desired cell number per well, leaving four wells without cells as background correction wells.

3 Add desired cells/well then centrifuge gently to adhere.

4 Gently add assay medium to each well: 100 uL for XF96 well plates and 315 µL for XF24 well plate. Total well volume should now be 150 µL for XF96 well plates and 415 µL for XF24 well plates.

5 Incubate the plate at 37 °C in a non-CO_2 incubator for 45 to 60 minutes prior to the assay.

6 Finally, just prior to starting the assay, add 30 µL of 1X Palmitate-BSA or 30 µL of 1X BSA control to the appropriate wells in 96-well plates. Alternatively, add 85 µL of 1X Palmitate-BSA or 85 µL of 1X BSA control to the appropriate wells in 24-well plates. The total volumes before the assay are 180 µL/well for 96-well plates and 500 µL/well for 24-well plates.

*Refer to the **Agilent Cell Analysis Learning Center** for details on preparation of suspension cells for XF assays.*

Prepare compound stock solutions and working solutions

CAUTION **Use compounds on the same day they are reconstituted. Do not freeze and reuse. Discard any remaining compound solutions.**

1 Remove one foil pouch and decapper from the XF Oxidation Stress Test kit box.

2 Open the pouch and remove all four vials: Oligomycin (blue cap), FCCP (yellow cap), Rotenone/Antimycin A (red cap) and Etomoxir (green cap).

3 Tap down the vials to ensure powder is on the bottom of the vial before removing the vial cap using the decapper provided with the kits.

4 Resuspend the content in each vial with the appropriate volume of prepared assay medium as described in **Table 11**. Vortex ~one minute to ensure full resuspension of compounds. These are compound stock solutions.

Table 11 Stock solutions.

Compounds from XF Long Chain Fatty Acid Oxidation Stress Test kit box	Volume of assay medium	Stock concentration
Etomoxir	700 µL	160 µM
Oligomycin	420 µL	150 µM
FCCP	720 µL	100 µM
Rotenone/Antimycin A	540 µL	50 µM

5 Use the compound stock solutions to prepare working solutions for loading into the injection ports on sensor cartridges.

6 Prepare 2 or 3 mL working solutions for each compound in Palmitate Oxidation Stress Test assay medium, using the volumes indicated in **Table 12** for XF/XFe96 sensor cartridges and **Table 13** for XF/XFe24 sensor cartridges. It is recommended to use 1.5 µM of oligomycin and 0.5 µM Rotenone/Antimycin A (final concentration) for most cells. Optimal FCCP concentration should be determined prior to the assay.

Table 12 Compound preparation for loading to XFe/XF96 sensor cartridges. Starting assay medium volume for cell plate is 180 L per well.

	Final well µM	Stock solution volume (µL)	Medium volume (µL)	10× (Port) µM	Volume added to port (µL)
Port A: Etomoxir	4	500	1,500	40	20
Port B: Oligomycin	1.5	300	2,700	15	22
Port C: FCCP	0.25	75	2,925	2.5	25
	0.5	150	2,850	5	25
	1.0	300	2,700	10	25
	2.0	600	2,400	20	25
Port D: Rotenone/Antimycin A	0.5	300	2,700	5	27

Table 13 Compound preparation for loading to XFe/XF24 sensor cartridges. Starting assay medium volume for cell plate is 500 L per well.

	Final well µM	Stock solution volume (µL)	Medium volume (µL)	10× (Port) µM	Volume added to port (µL)
Port A: Etomoxir	4	500	1,500	40	56
Port B: Oligomycin	1.5	300	2,700	15	62
Port C: FCCP	0.25	75	2,925	2.5	69
	0.5	150	2,850	5	69
	1.0	300	2,700	10	69
	2.0	600	2,400	20	69
Port D: Rotenone/Antimycin A	0.5	300	2,700	5	75

Load the injection ports on sensor cartridge

Proper port-loading techniques can be found on the **Agilent Cell Analysis Learning Center** website.

Please read the information prior to loading compounds. Ensure that the sensor cartridge is properly hydrated prior to use.

For the location of the ports, please refer to **Figure 9** on page 27.

Refer to **Table 14** for loading volume and port designation for compounds in different types of assays.

Table 14 Recommended injection volumes for Substrate Oxidation Stress Test injection schemes. Starting assay medium volume is 180 µL per well for 96-well cell plates and 500 µL per well for 24-well cell plates.

			Add to port volume	
Port	Compound	Port concentration	96 well	24 well
A	Etomoxir*	10×	20 µL	56 µL
B	Oligomycin	10×	22 µL	62 µL
C	FCCP	10×	25 µL	69 µL
D	Rotenone/Antimycin A	10×	27 µL	75 µL

* For controls, assay medium should be used in port A instead of inhibitors.

Load template onto the Seahorse XFe Analyzer

NOTE **If templates(s) are already present on the XFe analyzer, skip this step.**

Personal Computer (internet access required)

1 Use the hyperlink below to download the **Seahorse XF Substrate Oxidation Stress Test - Advanced Template** (for Palmitate Oxidation Stress Tests).
www.agilent.com/cs/library/software/public/software-agilent-seahorse-xf-subox-assay-template-files-cell-analysis-agilent.zip

2 Unzip the folder, then copy and paste the assay template files 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 Click **Import** (bottom of the New Assay view).

3 Locate the Assay Template file to import on the USB or network drive.

4 Click **Open** in the Windows dialogue box. The imported Assay Template will be available for selection from the Templates view on the XFe Analyzer.

5 Repeat for next template, if applicable.

6 The imported Assay Template(s) will now be available for selection in the list of available templates.

Running the XF Palmitate Oxidation Stress Test assay

1 Select the **Seahorse XF Palmitate Oxidation Stress Test-Advanced** template from the list of available templates, and click **Open File** (or double-click the template).

2 Group Definitions: confirm or modify the default groups and conditions for your assay.

3 Plate Map: confirm or modify the plate layout map for your assay.

4 Protocol: No action required - confirm or modify the Instrument Protocol for additional measurements cycles during the assay.

5 Run Assay: Click **Start Run** when ready.

6 When prompted, remove the cartridge lid, and place the loaded sensor cartridge with the utility plate on the thermal tray of the Seahorse XFe Analyzer. Ensure correct plate orientation and the cartridge lid has been removed. Then, click **I'm Ready**. Calibration takes approximately 15 to 30 minutes.

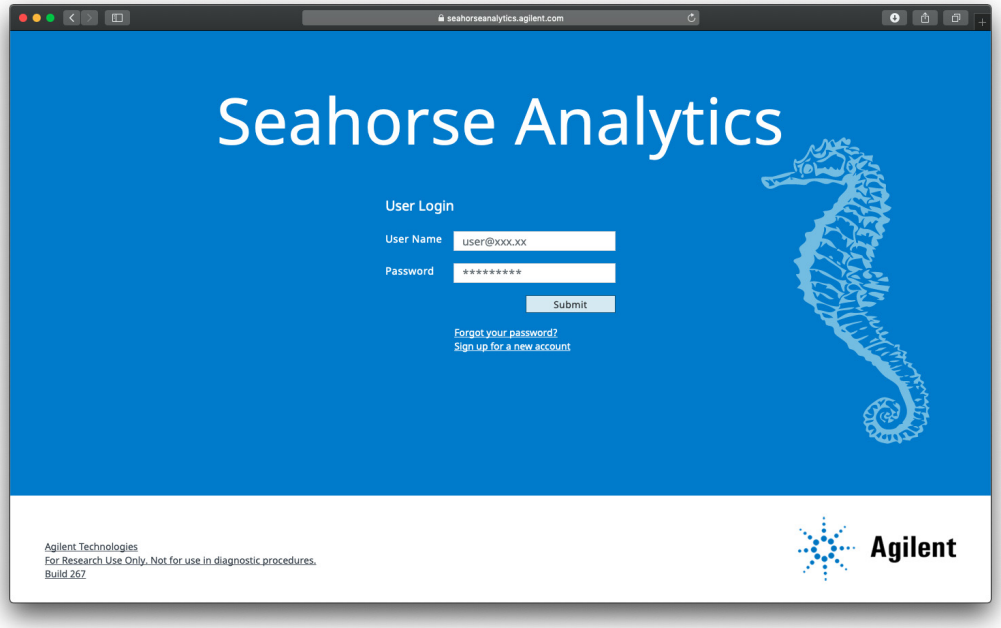
7 After completing Calibration, Wave Controller will display the Load Cell Plate dialog. Click **Open Tray** to eject Utility Plate and load the Cell Plate. Ensure the lid is removed from Cell Plate before Loading.

8 Click **Load Cell Plate** to run the assay.

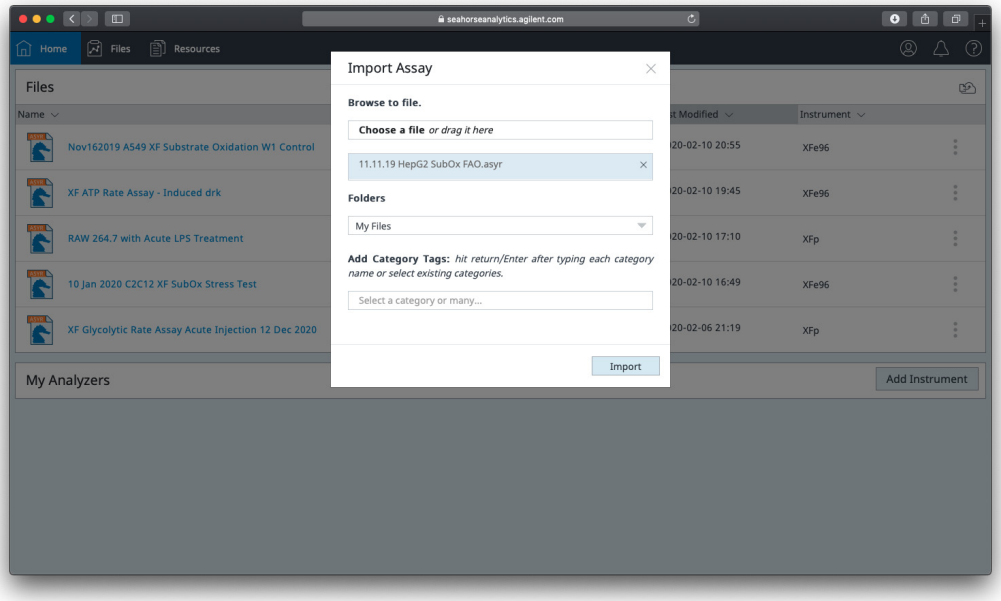
Data Analysis Using Agilent Seahorse Analytics

Agilent Seahorse Analytics is a new, web-based software platform that provides a simple, streamlined data analysis workflow for the XF Substrate Oxidation Stress Test assay. Seahorse Analytics automatically calculates the XF Palmitate Oxidation Stress Test parameters: basal respiration, acute response to inhibitor, and maximal respiration. Visit **https://seahorseanalytics.agilent.com** to register or log-in to your Seahorse Analytics account.

1. After the assay is completed, transfer your assay result file to your personal computer using a USB drive or network drive.
2. Go to **https://seahorseanalytics.agilent.com** to register or log in to your Seahorse Analytics account.

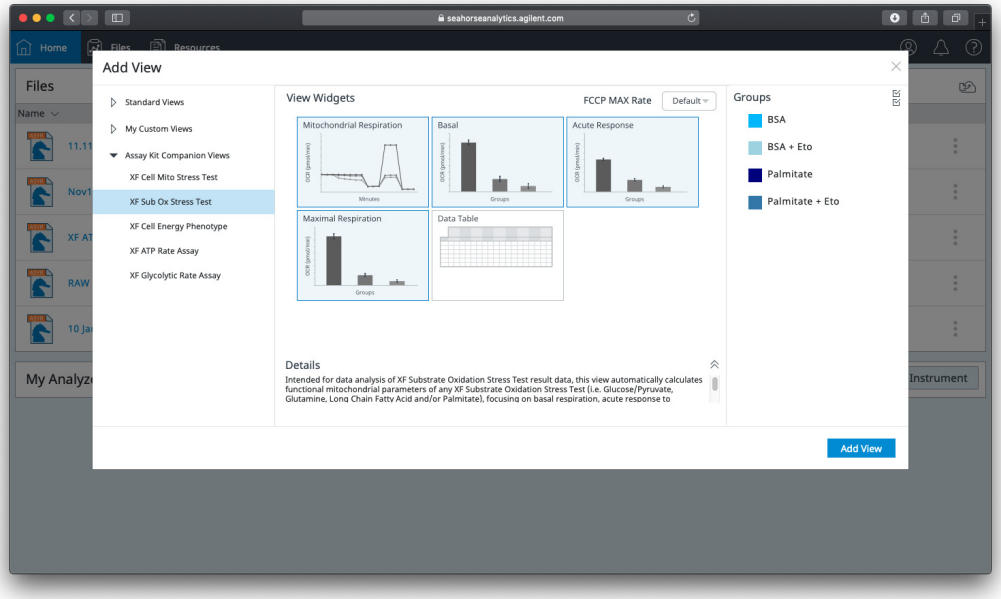


3 Import the assay result file to your account.



4 Open the assay result file and select the XF Substrate Oxidation Stress Test analysis view found under the Assay Kit Companion views menu.

5 Select groups to add to the analysis view, then click **Add View**.



Examples of Data

Figure 13 shows example Advanced Palmitate Oxidation Assay data from Seahorse Analytics for HepG2 cells.

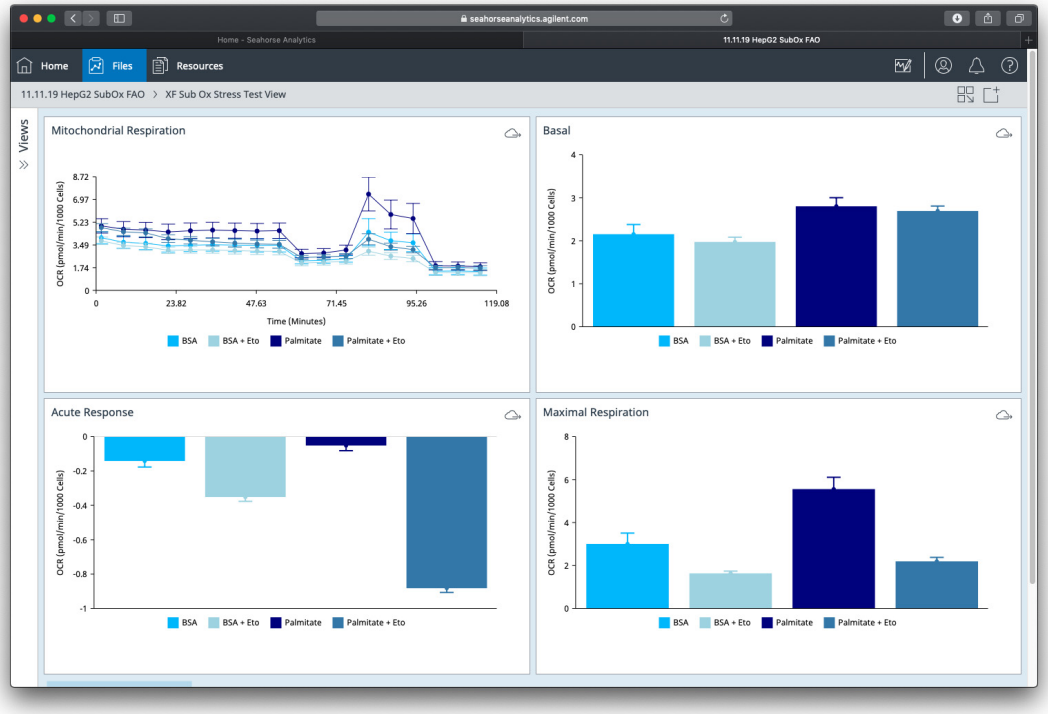


Figure 13. Advanced Palmitate Oxidation Assay data derived from Seahorse Analytics for HepG2 cells.

Figure 14 shows example data from experiments with A549 (Panel A) and HepG2 cells (Panel B).

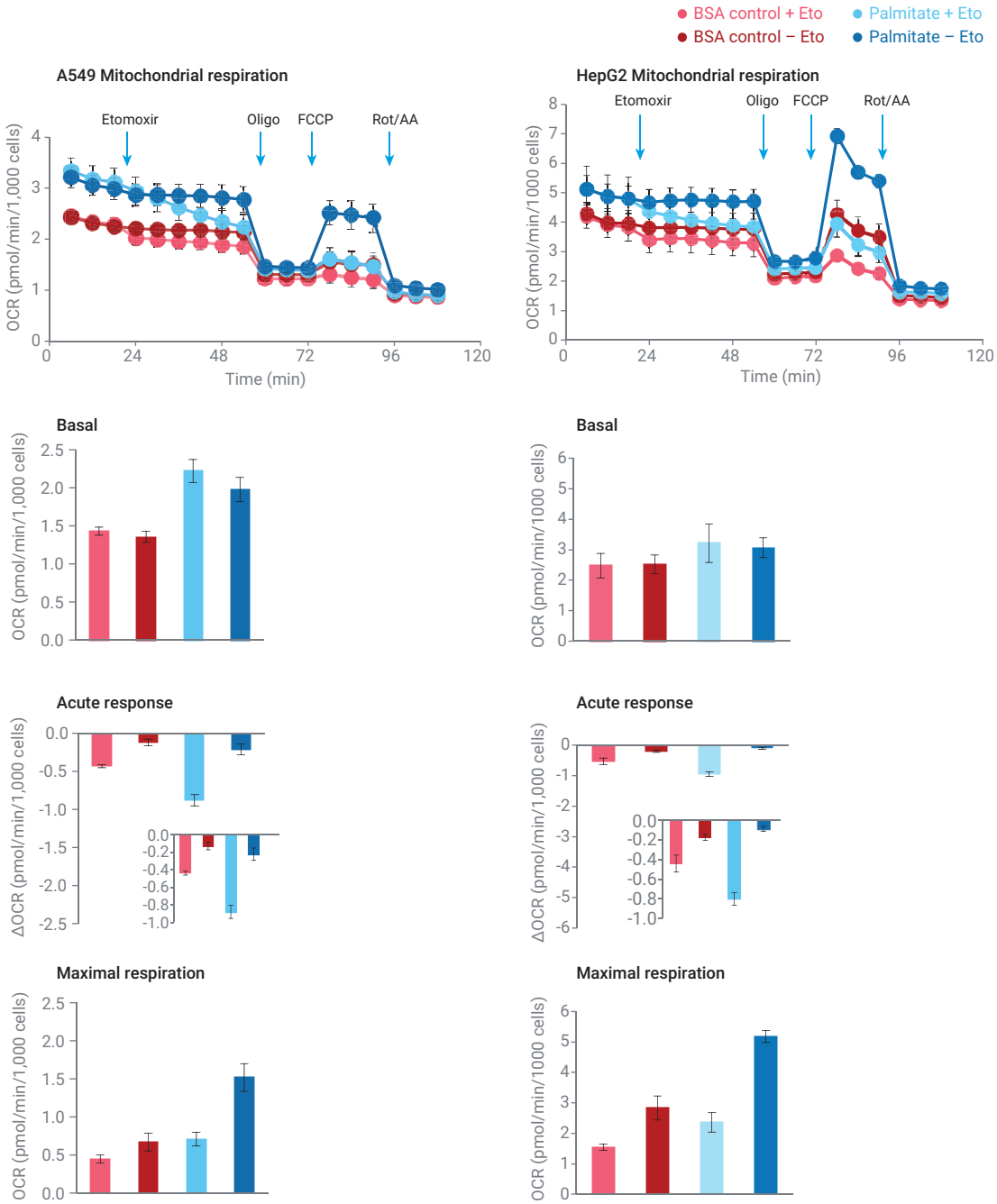


Figure 14. Palmitate oxidation in A549 and HepG2 cells using XF Palmitate Oxidation Stress Tests. A549 (A) and HepG2 (B) cells were seeded in XF96 Cell Culture Plates and grown overnight in appropriate cell growth media. The next day growth media was exchanged for substrate limited media (Table 1) and cultured for an additional 16 to 24 hours. Cells were then subject to XF Palmitate Oxidation Stress Tests using injections of assay media (control), or etomoxir (LCFA oxidation), followed by common sequential injections of oligomycin, FCCP, and Rtn/AA. XF Assay Media = XF DMEM, pH 7.4 + 2 mM glucose, + 0.5 mM L-carnitine. Each cell type was assayed on three individual days, with result data being compiled and processed through Seahorse Analytics. Error is reported as ± SEM. Similar results for both cell types tested were obtained using an XFe24 Analyzer (data not shown).

Inspection of the Basal Respiration parameter for each cell type shows the expected results for the given condition: the Palmitate groups show slightly increased OCR relative to BSA control groups, which are sensitive to etomoxir, with inhibition decreasing rates to those observed for the BSA control - Etomoxir groups (Rate 9, ~55 minutes). This indicates some palmitate is being oxidized in the basal (lower substrate demand) state. However, upon addition of FCCP, substrate demand is increased, and the Palmitate - Etomoxir groups show significant increases in maximal respiration compared to the groups with either BSA and/or +etomoxir. This indicates that these cell types not only have the ability to oxidize palmitate under the experimental conditions established, but that palmitate is the bulk of the substrate oxidized when forced to respire at a maximal rate (compare Palmitate ± Etomoxir groups). This data suggests that A549 and HepG2 cells are good candidate cell types for investigating how an intervention, such as genetic manipulation or drug exposure, applied to the cell specifically affect the oxidation of long chain fatty acids.

5 Frequently Asked Questions

Will these assays work for my cell type? Most likely. The basis of the Substrate Oxidation Stress Tests is the XF Cell Mito Stress, which has been used successfully with numerous cell types, including cell lines and primary cells. Please visit the **Agilent Cell Analysis Publication Database** to search for cell types used in publications.

Will these assays work for suspension, primary and/or immune, cells? Yes. However, please ensure proper experimental and assay procedures are followed for the cell type of interest.

Do I need to optimize cell seeding density? Yes. Please visit the XF Assay Learning Center and the Agilent Cell Analysis Publication Database for further information on basic cell density optimization procedures. Note that for the Advanced Palmitate Oxidation Stress Test, the cell density seeded may need to be decreased if cells are to be subject to substrate limitation, as this step requires an extra day of cell culture.

Do I need to optimize FCCP concentration? Yes. For the Standard Substrate Oxidation Stress Tests, this will likely be the same FCCP concentration used if the cell type of interest has been previously subject to a Cell Mito Stress Test.

NOTE

For the Advanced Palmitate Oxidation Stress Test, the concentration of FCCP may need to be increased due to the presence of BSA in the assay media. This should be performed before or during your initial Advanced Palmitate Oxidation Stress Test. Please visit the XF Assay Learning Center and the Agilent Cell Analysis Publication Database for further information on basic FCCP optimization procedures.

How do I know the inhibitors are at the correct concentration? The optimal concentration of each inhibitor is based on both testing at Agilent as well as information provided for etomoxir, UK5099 and BPTES in the following references, respectively: Divakaruni *et al.* 2018**1**; Hildyard *et al.* 2005**2**; and Robinson *et al.* 2007**3**.

What does it mean if there is no response to an inhibitor? Biologically, this suggests that the cell has no demand or reliance on that particular substrate under the experimental conditions defined. Note that it is common to see low to no responses to these inhibitors under basal respiration conditions (such as conditions of lower substrate demand).

Should I increase the concentration of the inhibitor? No. Each inhibitor is optimized for the correct final concentration: 4 µM for etomoxir, 2 µM for UK5099, and 3 µM for BPTES. Increased concentrations of inhibitors, especially etomoxir, will result in off-target effects, including effects on mitochondrial respiration.

What is the difference between the Long Chain Fatty Acid Oxidation Stress Test and the Palmitate Oxidation Stress Test? The Long Chain Fatty Acid Oxidation Stress Test is one of the three Standard Substrate Oxidation Stress Tests. This assay is focused on testing cellular demand of endogenous LCFAs under conditions of saturating substrates with respect to glucose, pyruvate and glutamine in the assay media, and best applied to answer questions about substrate demand and reliance with regard to LCFAO.

The Advanced Palmitate Oxidation Stress Test is designed to be performed under conditions of saturating palmitate (as Palmitate-BSA) and L-carnitine, with limited to no substrate provision with respect to glucose, pyruvate, and glutamine in the assay media. It is best applied when the experimental design calls for the cells to exclusively oxidize a long chain fatty acid, such as palmitate, to investigate effects of interventions specifically on the long chain fatty acid oxidation process.

When should I use Standard Assay workflow? The standard substrate oxidation assay is designed to be used when asking the following types of questions:

- Does the cell have a demand for a particular substrate or substrates?
- Is the cell highly reliant on a specific substrate, or can other substrates satisfy cellular demands?
- How is mitochondrial substrate demand and/or reliance affected if an intervention, such as a genetic manipulation or drug exposure, is applied to the cell?

What is the source of LCFAs for Standard LCFA assays? The source of long chain fatty acids are any endogenous stores of lipid/LCFAs in the cells used, and thus is cell-type dependent.

Can/Should I add Palmitate-BSA (source of FAs) to Standard Substrate Oxidation Stress Test assays? It is not recommended to add Palmitate-BSA to Standard Substrate Oxidation Stress Tests, as previous investigation has shown that cells typically will not respond to exogenous palmitate under conditions of fully supplemented cell culture media and subsequent saturating concentrations of glucose, pyruvate and glutamine in the XF assay media. No enhanced response to etomoxir has been demonstrated for cells supplemented with palmitate under these same conditions. For specific investigation of palmitate oxidation by cells, the Advance Assay for Palmitate Oxidation Stress Test is recommended

Can I alter the composition of the substrate oxidation stress test assay media (with regard to substrates)? It is recommended to begin the investigation with suggested substrate concentrations to establish cell behavior under saturating substrate concentrations. Final concentrations of supplements of glucose, pyruvate and glutamine may be altered, but care should be demonstrated when designing experiments and subsequent interpretation of data.

How is this different from the Seahorse XF Mito Fuel Flux Test? The XF Substrate Oxidation Stress Tests combine substrate pathway specific with the XF Cell Mito Stress Test (MST). Basal and maximal respiration rates are key metrics of mitochondrial function reported by the MST. In the context of substrate oxidation, the basal, and in particular, the maximal respiration rates are largely impacted by cells capacity to transport and oxidize available substrates[4]. This method is therefore ideally suited, and thus different from the mFFT, in that assessment for cellular substrate oxidation under conditions of both basal and elevated substrate demand (maximal respiration) where critical substrate dependence/reliance is more often revealed.

Do the Seahorse XF Mito Fuel Flex Test (mFFT) parameters (dependency, flexibility, capacity) apply here? No. The mFFT quantitative parameters do not apply to these assays. The well-recognized and accepted MST parameters (basal respiration, acute response and maximal respiration) are used for both Standard and Advanced Substrate Oxidation Stress Tests. Note, however, that terms such as substrate demand and reliance are used here qualitatively to describe cellular and mitochondrial function.

Can two (or more) inhibitors be injected at the same time? It is recommended to begin the investigation with the use of a single inhibitor to establish cell behavior. Two or more inhibitors can be applied at the same time to the same group of cells, but care should be taken when designing experiments and subsequent interpretation of data.

When should I use the Advanced Assay? This advanced assay is designed to be used when asking the following type of question: How an intervention, such as genetic manipulation or drug exposure, applied to the cell specifically affect the oxidation of long chain fatty acids?

What is the optimal nutrient deprivation media composition for my cell type (Advanced Procedure)? It is recommended to empirically determine the optimal nutrient deprivation media composition and time of exposure for the cell type of interest. Common parameters to test are decreased glucose, pyruvate, serum, and GlutaMAX concentrations in the substrate-limited growth media, as well as the length of time the cells are provided with this substrate-limited growth media before performing the assay.

Is the Palmitate saturating in concentration? Yes. The concentration of palmitate is saturating when used as recommended.

Can/Should I omit glucose from XF Palmitate Oxidation Stress Test assay media? The final glucose concentration in the XF Palmitate Oxidation Stress Test assay media should be empirically tested. Complete removal of glucose is not recommended as low concentrations of glucose are required by the cell to ensure proper function of glycolysis and the TCA cycle, which are interrelated to and can impact optimal oxidation of palmitate.

Can the Advanced Assay be applied to glucose/pyruvate and/or glutamine oxidation? Currently, the Advanced Assay method is validated only for use with the Palmitate Oxidation Stress Test Kit. While it is possible to apply the Advanced Assay method to specifically investigate glucose/pyruvate, and/or glutamine oxidation in the absence of other substrates, the potential interference between glucose and glutamine demand can be substantial when lowering either or both to sub-saturating levels in the substrate-limited growth and/or substrate-limited assay media. Initial suggestions for optimization of advanced experimental conditions would include cross titrations of glucose and glutamine concentrations across a range of both saturating and subsaturating values for primary analysis.

6 Context of Substrate Oxidation Tests Among Other XF Kits and Applications

Agilent offers a variety of XF Assay Kits that provide a full spectrum of information, from broad assessment of cellular function down to specific details of metabolic mechanism (**Figure 15**). Beginning with the XF Real-Time ATP Rate Assay, consider this as a compass that can point to or uncover phenotypic shifts, metabolic switching, pathway liabilities, and more, through basic information about cellular energetic phenotype. This assay also indicates a suitable follow-up XF assay to delve deeper in metabolic mechanisms and/or the influence of a specific pathway on cellular function. For example, the XF Cell Mito Stress Test and XF Glycolytic Rate Assay are designed to interrogate mitochondrial and glycolytic function, respectively, in the context of the entire cell. Once a change in mitochondrial function has been identified, often the next steps in the investigation are to understand what may be responsible for this change, including the effects of oxidation of glucose/pyruvate, glutamine, and long-chain fatty acids. Subsequently, this information can be used to develop strategies for control of cell function and phenotype through metabolic programming and engineering.

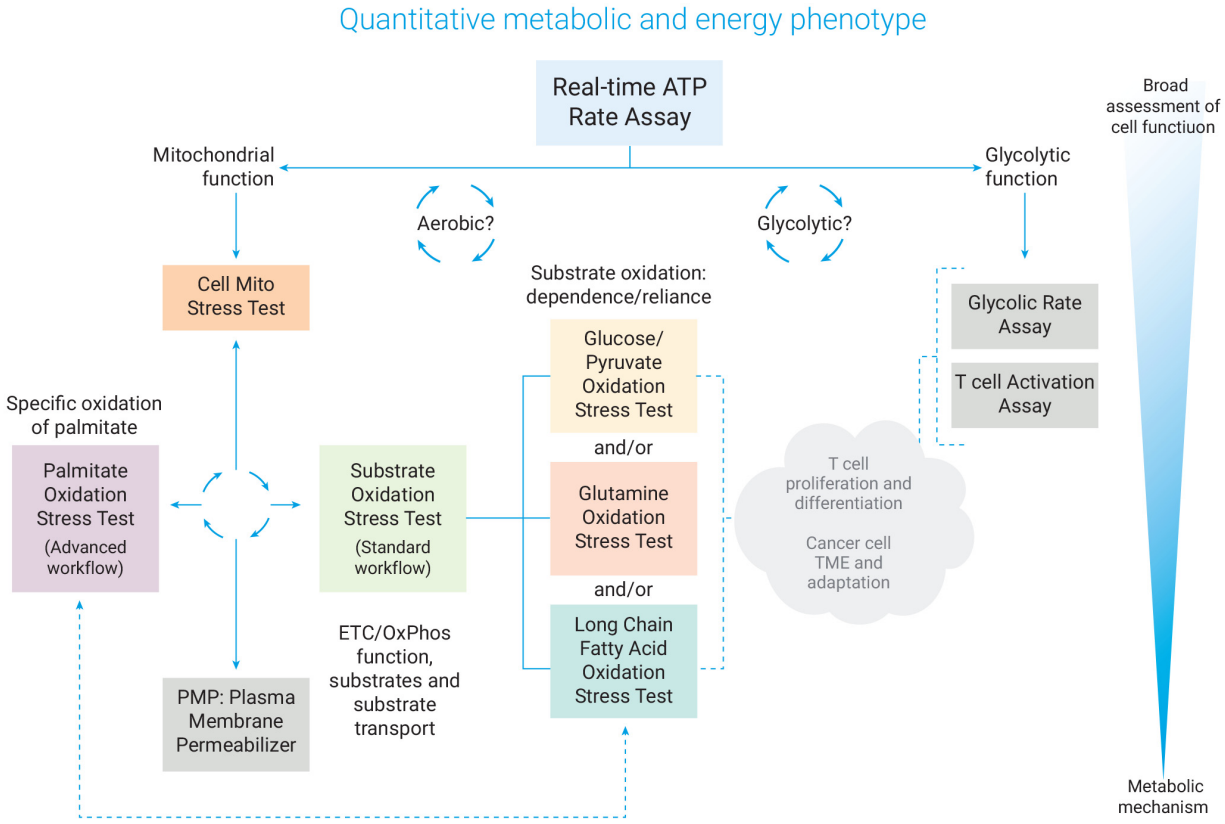


Figure 15. Agilent offers a variety of XF Assay Kits that provide a full spectrum of information, from broad assessment of cellular function down to specific details of metabolic mechanism. Once a change in mitochondrial function has been identified, the next steps in the investigation are to understand what may be responsible for this change, including the effects of oxidation of glucose/pyruvate, glutamine, and long chain fatty acids.

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