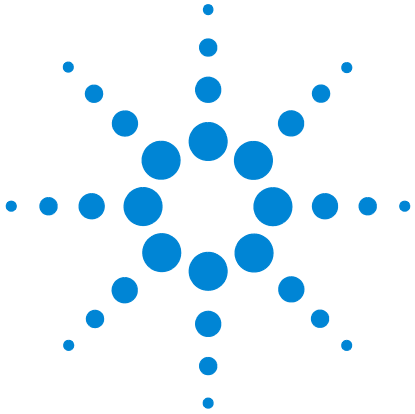

Agilent Seahorse XF Glycolytic Rate Assay Kit

User Guide
Kit 103344-100

Agilent Technologies

Notices

Manual Part Number

103344-400

Kit Part Number

103344-100

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Contents

1 Introduction

Assay Background

For use with Agilent Seahorse XF96, XFe96 and XFe24 Extracellular Flux Analyzers

The Agilent Seahorse XF Glycolytic Rate Assay is an accurate and reliable analytical method for measuring glycolysis in cells. Together with a Seahorse XFe24, XF96 or XFe96 Analyzer, the Seahorse XF Glycolytic Rate Assay provides accurate measurements of glycolytic rates for basal conditions and compensatory glycolysis following mitochondrial inhibition (see Figure 1 on page 6). The calculated rates account for contribution of CO_2 to extracellular acidification derived from mitochondrial/TCA cycle activity and are directly comparable to lactate accumulation data.

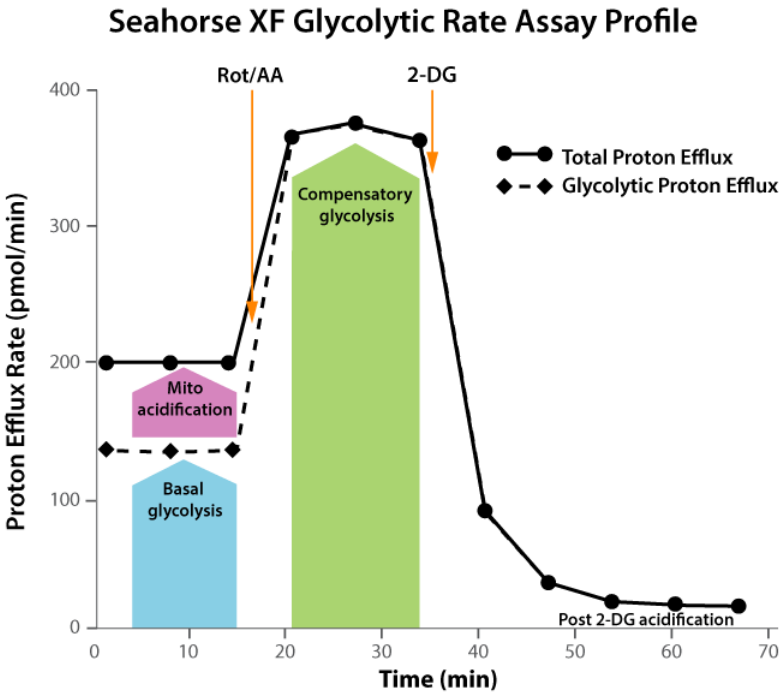

Figure 1 Agilent Seahorse XF Glycolytic rate assay profile. Proton efflux from live cells comprises both glycolytic and mitochondrial-derived acidification. Inhibition of mitochondrial function by Rotenone and Antimycin A (Rot/AA) enables calculation of mitochondrial-associated acidification. Subtraction of mitochondrial acidification to Total Proton Efflux Rate results in Glycolytic Proton Efflux Rate.

Seahorse XF Analyzers directly measure real time extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cells - indicators of the two major energy-producing pathways: glycolysis and oxidative phosphorylation. Most cells possess the ability to switch between these two pathways, thereby adapting to changes in their environment. To measure glycolytic rates, Seahorse XF Glycolytic Rate Assay utilizes both ECAR and OCR measurements to determine the glycolytic proton efflux rate (glycoPER) of the cells (defined below).

Glucose in cells is converted to pyruvate, and then converted to lactate in the cytoplasm, or to CO_2 and water in the mitochondria. The conversion of glucose to lactate results in a net production and extrusion of protons into the extracellular medium (see Figure 2 on page 8).

Cells may also use glucose and the other fuels present within the cell or in the assay medium for energy production through mitochondrial respiration. Mitochondrial-derived CO_2 can partially hydrate in the extracellular medium, resulting in additional extracellular acidification beyond that contributed by glycolysis. By also measuring the amount of oxygen consumed by the cell, the contribution of mitochondria/CO_2 to extracellular acidification is calculated and used to subtract the CO_2 contributing acidification from the total Proton Efflux Rate (PER). (For a detailed explanation, please read the *Agilent Seahorse XF CO_2 Contribution Factor Protocol User Guide*.) The resulting value, glycoPER, is the rate of protons extruded into the extracellular medium during glycolysis. This assay allows for real-time measurements of changes in glycolysis rates that may go undetected in long-term lactate accumulation assays.

The assay workflow is as follows: first, cells are incubated in the Seahorse XF Glycolytic Rate Assay Medium containing substrates such as glucose, glutamine, and pyruvate, as well as HEPES buffer, and basal rates are recorded over three measurement periods. Next, Rot/AA (inhibitors of mitochondrial electron transport chain) are injected to inhibit mitochondrial oxygen consumption (and therefore CO_2- derived protons). The second injection is 2-deoxy-D-glucose (2-DG), a glucose analog which inhibits glycolysis through competitive binding of glucose hexokinase, the first enzyme in the glycolytic pathway. The resulting decrease in PER provides qualitative confirmation that the PER produced prior to the injection is primarily due to glycolysis.

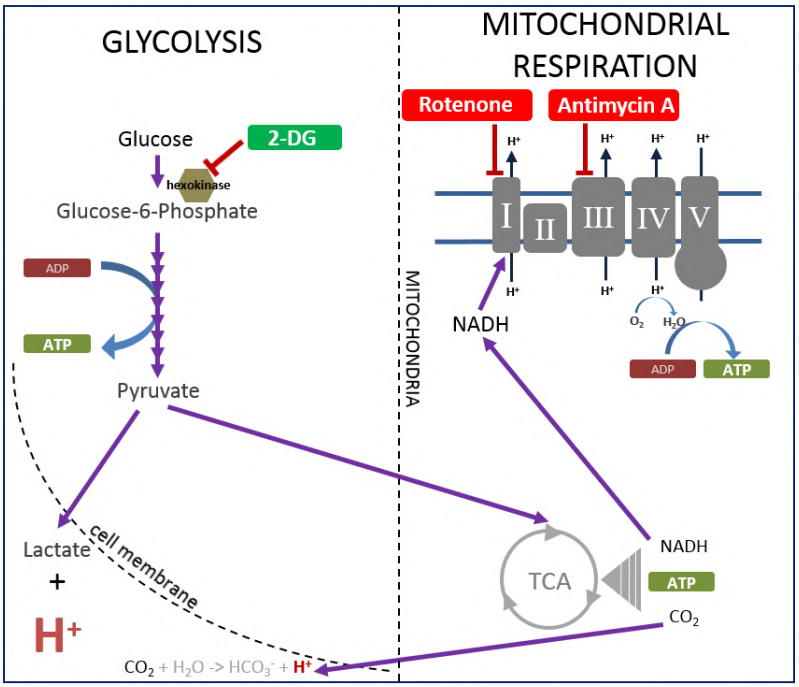

Figure 2 Principle of the Agilent Seahorse XF Glycolytic rate assay. Energy is produced by two pathways in the cell - glycolysis and mitochondrial respiration. In the breakdown of glucose to lactate during glycolysis, protons are extruded into the extracellular media which is detected by the XF Analyzer as ECAR. Additionally, mitochondrial TCA activity produces CO_2, which hydrates and acidifies the media. By inhibiting respiration (OCR) during the assay with the complex I and complex III mitochondrial inhibitors (Rot/AA), the rate of proton efflux from respiration can be calculated and removed from the total proton efflux rate giving the glycoPER. To confirm pathway specificity, 2-DG, an inhbitor of glycolysis is injected to stop glycolytic acidfication.

Glossary

- **Glycolysis:** In the context of the Seahorse XF Glycolytic Rate Assay, the process of converting glucose to lactate.
- **Buffer factor (BF):** Buffer capacity of the measurement system, comprising the assay medium and XF assay conditions (instrument, sensor, labware).
- **Proton efflux rate (PER):** The number of protons exported by cells into the assay medium over time, expressed as pmol/min.
- **Glycolytic proton efflux rate (glycoPER):** Proton Efflux rate derived from glycolysis (discounting the effect of CO_2-dependent acidification). This measurement is highly correlated with the extracellular lactate production rate.
- **Compensatory glycolysis:** The rate of glycolysis in cells following the addition of mitochondrial inhibitors, effectively inhibiting oxidative phosphorylation and driving compensatory changes in the cell to use glycolysis to meet the cells' energy demands.
- **Post-2-DG acidification:** This value includes other sources of extracellular acidification that are not attributed to glycolysis or mitochondrial TCA activity as well as any residual glycolysis not fully inhibited by 2-DG. It is measured after the addition of 2-DG in the Glycolytic Rate Assay workflow.
- **Induced assay:** Assay workflow that includes an acute injection of an experimental compound prior to injection of XF Glycolytic Rate Assay compounds. This workflow allows for real-time quantification of *in situ* glycolysis activation or repression.

2 Kit Information

Kit Contents

The Seahorse XF Glycolytic Rate Assay Kit includes six foil pouches, each containing Rot/AA mix and 2-DG. The kit reagents are sufficient for six complete XF Glycolytic Rate Assays in a 96 or 24-well Seahorse XF Cell Culture Microplate.

Table 1 Agilent Seahorse XF Glycolytic Rate Assay kit contents in each foil pouch

Compound	Target	Effect	Cap color	Quantity per tube
Rotenone plus Antimycin A (Rot/AA)	Mitochondrial ETC complexes I and III, respectively	Inhibits mitochondrial respiration, typically leading to an increase in glycolysis	Red	27 nmol each
2-deoxy-D-glucose (2-DG)	Hexokinase (rate-limiting enzyme in glycolysis)	Inhibits hexokinase, leading to a decrease in glycolysis	Green	1,500 µmol

Kit Shipping and Storage

The product ships at ambient temperature, and should be stored at room temperature. The product is stable for one year from the date of manufacture. The actual expiration date is printed on the label of the assay kit box. Depending on the shipping date, the actual shelf life of the kit in user's hand can vary between 12 to 3 months.

Additional Required Items

The following items are required to run the Seahorse XF Glycolytic Rate Assays, but not supplied with the kit.

Items	Supplier	Catalog number
Agilent Seahorse XFe/XF Analyzers	Agilent Technologies	
For XFe/XF96 Analyzers: XFe96 FluxPak mini or XFe96 FluxPak	Agilent Technologies	 102601-100 or 102416-100
For XFe24 Analyzers: XFe24 FluxPak mini or XFe24 FluxPak	Agilent Technologies	 102342-100 or 102340-100
XF DMEM medium, pH 7.4* or XF RPMI medium, pH 7.4*	Agilent Technologies	103575-100 103576-100
XF 1.0 M Glucose solution	Agilent Technologies	103577-100
XF 100 mM Pyruvate solution	Agilent Technologies	103578-100
XF 200 mM Glutamine solution	Agilent Technologies	103579-100

* XF DMEM or RPMI media can also be purchased together with the supplements listed in this table as bundled products (Catalog Number 103680-100 and 103681-100). For a full list of all medium types and our recommendation for each assay kit, please refer to the Seahorse XF Media Selection Guide.
http://www.agilent.com/cs/library/selectionguide/public/5991-7878EN.pdf

Narrow p1000 pipette tips are recommended for reconstituting compounds within the tubes provided (for example, Fisherbrand SureOne Micropoint Pipet Tips, catalog #: 02-707-402).

3 Assay Workflow

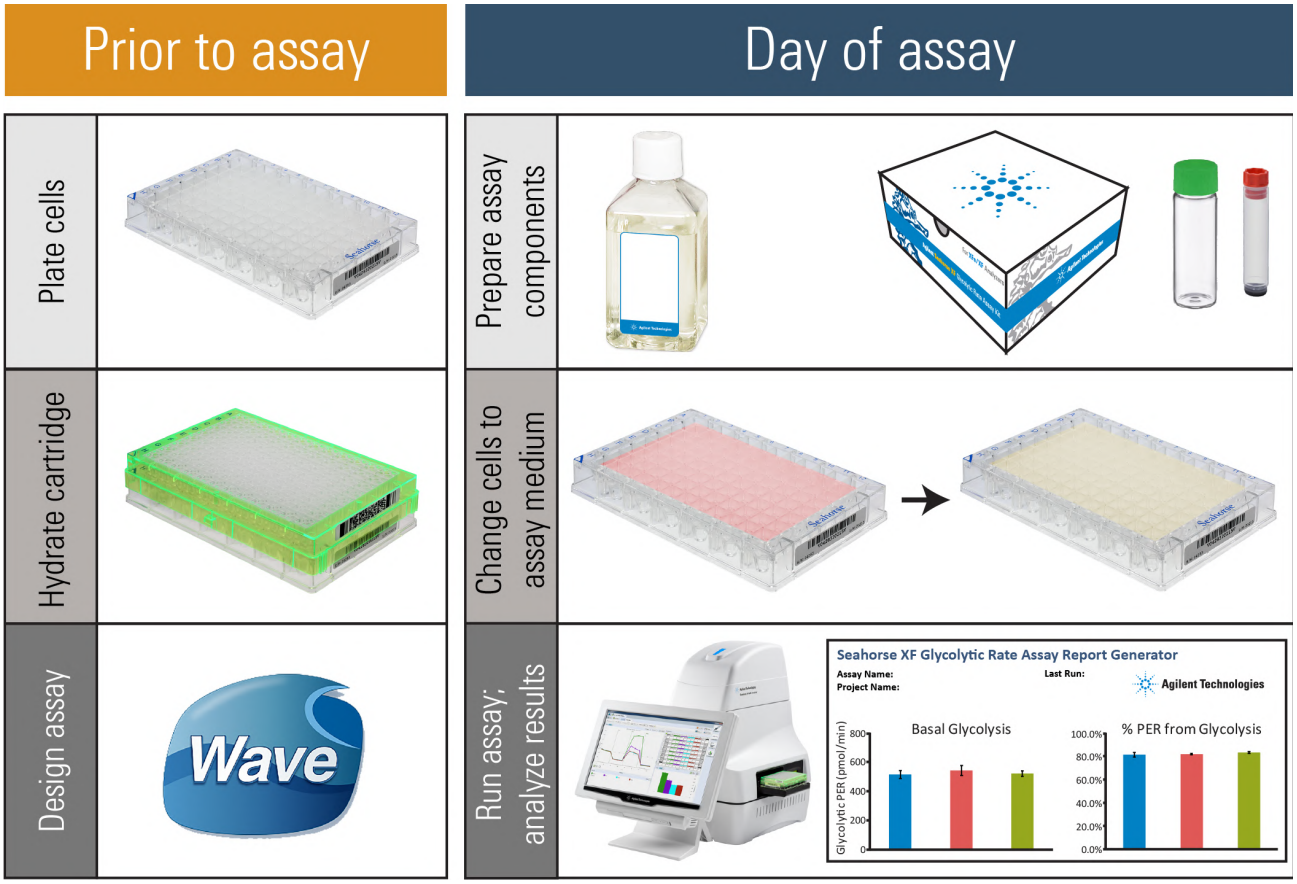

Figure 3 Agilent Seahorse XF Glycolytic Rate Assay Workflow

Day Prior to Assay

1 Turn on the Seahorse XFe/XF96 or XFe24 Analyzer, and allow the temperature to stabilize.

2 For adherent cells, plate cells at a predetermined density in the Agilent Seahorse XF Cell Culture Microplate using the appropriate cell culture growth medium.
For more information, refer to the Basic Procedure, “Seeding Cells in Seahorse XF Cell Culture Microplates”, available on the Agilent Cell Analysis Learning Center.
www.agilent.com/en/products/cell-analysis/how-to-run-an-assay

NOTE

The Cell Line Reference Database is a good resource for finding information regarding the cell type of interest. Use the link below to obtain information.

http://www.agilent.com/cell-reference-database

3 Hydrate a sensor cartridge in Seahorse XF Calibrant at 37 °C in a non-CO_2 incubator overnight (refer to Basic Procedure).

4 For XFe analyzers, load the XF Glycolytic Rate Assay Template in Wave. Make any necessary group modifications to tailor the template to the specific assay design.

Day of Assay

Prepare Agilent Seahorse Glycolytic rate assay medium

1 Prepare assay medium by supplementing Seahorse XF DMEM or RPMI medium with pH 7.4 (Cat No 103575-100 and 103576-100). These media contain the appropriate amount of HEPES and NO additional HEPES is needed. It is recommended to start with 1 mM pyruvate,
2 mM glutamine, and 10 mM glucose. However, medium composition can be changed depending on cell type or the desired study conditions. For more information, refer to the Basic Procedure, “Preparing Assay medium for Use in XF Assays”, on the Agilent Cell Analysis Learning Center.
www.agilent.com/en/products/cell-analysis/how-to-run-an-assay

NOTE

If the assay medium is significantly changed from this formulation, the Buffer Factor Protocol must be performed to derive the buffer factor value. Consult *Agilent Seahorse XF Buffer Factor Protocol User Guide* for more information.

2 Bring XF medium with pH 7.4 and XF supplements into a cell culture hood. Transfer a sufficient volume to a sterile bottle. It is not necessary to warm the medium and supplements before this step.

3 Add proper volumes of XF supplements to achieve the desired final concentrations. This is your assay medium. When recommended supplement concentrations are used, pH-adjustment is not necessary.

4 Warm the assay medium to 37 °C in a water bath. It is ready to use.

Prepare Agilent Seahorse XF Cell culture microplate for assay

For adherent cells

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

2 Wash the cells (refer to Basic Procedures to Run an XF assay for more details). Remove the cell culture growth medium in the cell culture microplate. Wash once with warmed assay medium using a multichannel pipette, and incubate with assay medium at 37 °C in a non-CO_2 incubator for 45-60 minutes prior to the assay.

3 Before starting the XF assay, AGAIN remove the assay medium and add fresh, warm assay medium (see Table 4 on page 17 for appropriate Starting Well Volume).

For suspension cells

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

2 Count the cells, and suspend them 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 the desired cells/well then centrifuge gently to adhere.

4 Gently add assay medium to each well. Total well volume should match the appropriate Starting Well Volume as indicated in Table 4 on page 17.

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

Prepare stock compounds

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

1 Remove one foil pack from the kit box, then open the pouch, and remove the Rot/AA (red cap) vial and one 2-DG (green cap) vial.

2 Tap down the vials to ensure the powder is on the bottom of the tube before opening the vials.

3 Resuspend each component with prepared assay medium as described in Table 2 with a p1000 pipette. Tap down Rot/AA to ensure powder is on the bottom of the tube. Vortex ~1 minute to ensure that compounds go into solution.

Table 2 Stock solutions

Compound	Volume of assay medium	Resulting stock concentration
Rot/AA	540 µL	50 µM
2-DG	3000 µL	500 mM

Dilute compounds

Table 3 describes how to prepare compounds dilutions to load the cartridges. Please note that if different starting assay volumes or port volumes are used, adjust compounds concentrations in order to obtain the recommended final concentrations in the well.

Table 3 Compound preparation for running the XF Glycolytic Rate Assay on an XF96, XFe96, or XFe24 Analyzer

Port A Rot/AA	(Final well) (µM)	Stock volume (µL)	Medium volume (µL)	10X (Port) (µM)
	0.5	300	2700	5
Port B 2-DG	**(Final well) (mM)**	**Stock volume (µL)**	**Medium volume (µL)**	**10X (Port) (mM)**
	50	3000	0	500

Load sensor cartridge

Standard assay - No injection before Glycolytic Rate Assay compounds. Load compounds into the following ports of a hydrated sensor cartridge:

- Port A: Rot/AA
- Port B: 2-DG

Induced assay -To inject a test compound prior to the Glycolytic Rate Assay compounds, use port A for the desired compound and then load ports as follows:

- Port A: experimental compound (acute injection) or media control
- Port B: Rot/AA
- Port C: 2-DG

Table 4 lists the appropriate volumes and concentrations for injection schemes using two or more ports.

Table 4 Starting well assay medium volume and compound injection volumes

	Agilent Seahorse XFe/XF96 Analyzer		Agilent Seahorse XFe24 Analyzer	
	Starting well volume: 180 µL assay medium		Starting well volume: 500 µL assay medium	
Port	Vol.	Conc.	Vol.	Conc.
A	20 µL	10X	56 µL	10X
B	22 µL	10X	62 µL	10X
C	25 µL	10X	69 µL	10X
D	27 µL	10X	76 µL	10X

Running the Assay

Load template onto the Seahorse XFe Analyzer

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

Personal Computer (internet access required):

1 Download the Seahorse XF Glycolytic Rate Assay Report Generator from the Agilent website. Both XFe Glycolytic Rate Assay Basic and Induced 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 USB drive in 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.

5 Repeat for next template, if applicable.

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

Run the Seahorse XF Glycolytic Rate Assay

1. Select the **Seahorse XF Glycolytic Rate Assay** or **Seahorse XF Glycolytic Rate Assay (Induced Assay)** template from the list of available templates and click **Design** (or double-click the template).
2. Groups/Conditions: No action required – confirm or modify the default groups and conditions for your assay.
3. Plate Map: No action required – confirm or modify the Plate Map for your assay.
4. Instrument Protocol: No action required – confirm or modify the Instrument Protocol for additional measurements cycles during the assay.
5. Review and Run: Click **Start Run** when ready.
6. When prompted, place the loaded sensor cartridge with the calibrant plate into the Seahorse XFe Analyzer, then click **I'm Ready**. Calibration will take approximately 15-30 minutes.

NOTE Remove Cartridge lid and verify correct plate orientation

7. Click **I'm Ready** after Calibration to load the cell culture microplate.
8. Press **I'm Ready** to close the tray door and begin the assay.

Analysis Using the Agilent Seahorse Glycolytic Rate Assay Report Generator

1. From Wave, export the data from the completed run as an .xls file.
2. Load the data file in the XF Glycolytic Rate Assay Report Generator, and select groups to display.
3. Click **Update Summary** to obtain the XF Glycolytic Rate Assay Report. For further details, consult the *Agilent Seahorse XF Glycolytic Rate Assay Report Generator User Guide*.

4 Frequently Asked Questions

Why does the assay require the use of buffered media, will it dampen the ECAR signal?

A low concentration of HEPES (5 mM) has been found to provide consistent buffer capacity values across the time frame of the assay. Although this low concentration of HEPES may reduce the raw ECAR signal slightly, it significantly improves the consistency of the ECAR signal as well as the accuracy of the transformation to PER.

Does acidification of the media by CO_2 effect the ECAR measurements?

Acidification of the media by CO_2 produced from the TCA cycle can affect ECAR, but its relative contribution varies widely among cell types. By measuring OCR before and after the Rot/AA injection, and using the Buffer Factor (BF) and CO_2 contribution factor (CCF), the mitochondrial PER is calculated and subtracted to generate the glycoPER. The Seahorse XF Glycolytic Rate Assay reports the percentage of acidification coming from glycolysis as an easy indicator of whether a substantial amount of acid is coming from CO_2.

How do I calculate mitochondrial acidification from mitochondrial oxygen consumption rate?

There is a linear correlation between mitochondrial oxygen consumption rate (mitoOCR) and mitochondria-derived acidification (mitoPER), being the CO_2 Contribution Factor (CCF), the ratio between mitoPER/mitoOCR, a constant among most cell types. During Glycolytic Rate Assay post-run analysis using a Seahorse XF Glycolytic Rate Assay Report Generator, mitochondrial contribution to acidification is calculated from mitoOCR using the predetermined CCF. However, for cells that are highly oxidative (% PER from glycolysis is <50%) we recommend to reconfirm CCF for the specific cell type using

Seahorse XF CO_2 Contribution Factor Protocol User Guide. For more details about calculations and derivation of constants see Agilent White Paper *Improving Quantification of Cellular Glycolytic Rate Using Seahorse XF Technology* http://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00ca/t/page/fm/1.

What information does this assay give me that looking at basal ECAR cannot?

Whereas basal ECAR is a good qualitative indicator of glycolysis in most circumstances, it includes acidification of the media from any and all acid sources, and does not consider the buffering properties of the assay medium. The Seahorse XF Glycolytic Rate Assay provides a more precise measurement of extracellular acidification specifically due to glycolysis by subtracting out mitochondrial sources of acidification, as well as reporting the data in standard units (pmol/min). These features make the Seahorse XF Glycolytic Rate Assay highly comparable to extracellular lactate production measurement assays.

Do I have to calculate the buffer factor (BF) myself?

No- if you are using the recommended formulation. The Buffer Factor has been predetermined for the standard XF Seahorse Glycolytic Rate Assay medium described above. When using an assay medium with an alternative composition (different base medium or concentrations of substrates), then the buffer factor should be determined empirically in the system following the *Seahorse XF Buffer Factor Protocol*.

Do I have to use phenol-red free medium? Why?

Phenol red interferes with the pH sensor, causing an apparent pH lower than the actual pH of the assay medium. While this does not affect raw ECAR values, to accurately calculate PER and glycoPER, phenol red must be omitted from the assay media.

Questions: Contact Seahorse Technical Support:

Email: Seahorse.support@agilent.com

Ph: 800-227-9770, option 3, option 8

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