Agilent Seahorse XF Real-Time ATP Rate Assay Kit

User Guide
Kit 103592-100

For use with Seahorse XF Pro, XFe96 and XFe24 Analyzers.

Notices

© Agilent Technologies, Inc. 2024

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

103592-400 Rev C

Kit Part Number

103592-100

Edition

Second edition, December 2024

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Boulevard
Santa Clara, CA 95051

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data -Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A CAUTION notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a CAUTION notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

Contents

1 Introduction

Assay Background

The cellular rate of adenosine triphosphate (ATP) production is a highly informative measurement to describe cellular metabolism, as ATP is the ubiquitous, dominant energy currency for cells. Cellular metabolic regulation allows cells to adjust for changes in ATP demand with subsequent changes in ATP production to maintain total intracellular ATP levels.

The Agilent Seahorse XF Real-Time ATP Rate Assay is designed to measure total ATP production rates in living cells. Even more relevant is the ability of this assay to distinguish between the fractions of ATP that are produced from mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis, the two main metabolic pathways responsible for ATP production in mammalian cells.

The Seahorse XF Real-Time ATP Rate Assay uses metabolic modulators (oligomycin and a mix of rotenone and antimycin A, see **Figure 1** on page 6) that when serially injected, allow the calculation of the mitochondrial and glycolytic ATP production rates. Together with a Seahorse XFe24, XFe96 or XF Pro Analyzer, the Seahorse XF Real-Time ATP Rate Assay provides a new dynamic and quantitative insight into cellular bioenergetics by providing a real-time measurement of cellular ATP production rates and a quantitative phenotype of cellular energy poise.

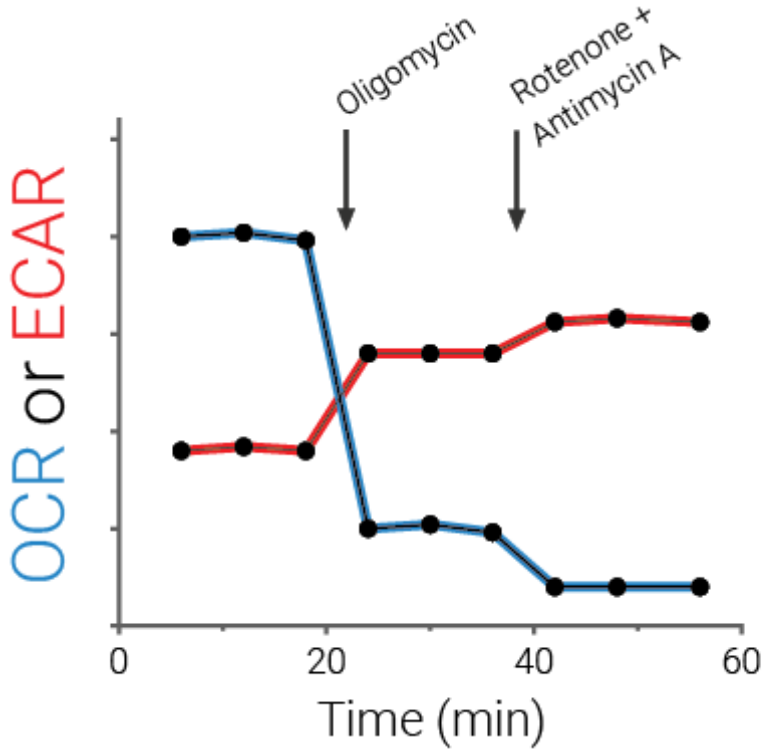

Figure 1. Representative scheme of Agilent Seahorse XF Real-Time ATP Rate Assay - Kinetic profile of OCR and ECAR measurements.
Basal OCR and ECAR rates are first measured. Injection of oligomycin results in an inhibition of mitochondrial ATP synthesis that results in a decrease in OCR, allowing the mitoATP Production Rate to be quantified. ECAR data combined with the buffer factor of the assay medium allows calculation of total Proton Efflux Rate (PER). Complete inhibition of mitochondrial respiration with rotenone plus antimycin A allows accounting for mitochondrial-associated acidification, and when combined with PER data allows calculation of the glycoATP Production Rate.

In mammalian cells, glycolysis and oxidative phosphorylation (OXPHOS) pathways provide the majority of cellular ATP. While OXPHOS consumes O_2, driving the oxygen consumption rate (OCR), both pathways can contribute to the acidification of the assay medium. Conversion of glucose to lactate through glycolysis is accompanied by extrusion of one H^+ per lactate, while the TCA cycle

that fuels ETC/OXPHOS produces CO_2, which also results in acidification of the assay medium. The sum of these reactions is the primary driver of changes in extracellular acidification (ECAR).

Seahorse XF technology measures the flux of both H^+ production (ECAR) and O_2 consumption (OCR), simultaneously. By obtaining these data under basal conditions and after serial addition of mitochondrial inhibitors (oligomycin and rotenone/antimycin A), total cellular ATP Production Rates and pathway-specific mitoATP and glycoATP Production Rates can be measured label-free and in real time. The series of calculations used to transform the OCR and ECAR data to ATP Production Rates is described below and are performed post data acquisition using Seahorse Analytics.

Glycolytic ATP production rate calculation

During conversion of one molecule of glucose to lactate in the glycolytic pathway, 2 molecules each of ATP, H+ and lactate are produced (Equation 1):

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{Pi} \rightarrow 2\ \text{Lactate} + 2\ \text{ATP} + 2\ H_2O + 2\ H^+ \quad \textbf{(Equation 1)}$$

Considering the stoichiometry of the glycolytic pathway (**Equation 1**), the rate of ATP production in the glycolytic pathway (glycoATP Production Rate) is equivalent to Glycolytic Proton Efflux Rate (glycoPER, **Equation 2**) and can be calculated using the same approach previously validated for the Agilent Seahorse XF Glycolytic Rate Assay (for more details see **White paper: Improving Quantification of Cellular Glycolytic Rate Using Agilent Seahorse XF Technology**)

$$\text{glycoATP Production Rate (pmol ATP/min)} = \text{glycoPER (pmol } H^+\text{/min)} \quad \textbf{(Equation 2)}$$

Mitochondrial ATP production rate calculation

The rate of oxygen consumption that is coupled to ATP production during OXPHOS can be calculated as the OCR that is inhibited by addition of the ATP synthase inhibitor, oligomycin (**Equation 3**):

$$OCR_{ATP}\ (\text{pmol } O_2\text{/min}) = \text{OCR (pmol } O_2\text{/min)} - OCR_{Oligo}\ (\text{pmol } O_2\text{/min}) \quad \textbf{(Equation 3)}$$

Transformation of OCR_{ATP} to the rate of mitochondrial ATP production consists of: multiplying by 2 to convert molecules of O_2 to oxygen (O) atoms consumed, then multiplying the P/O ratio, the number of molecules of ADP phosphorylated to ATP per atom of O reduced by an electron pair flowing through the electron transfer chain (**Equation 4**).

The Seahorse XF Real-Time ATP Rate Assay uses an average P/O value of 2.75 for these calculations that was validated to accurately represents the assay conditions for a broad panel of cells under different fuels availabilities (see **White Paper: Quantifying Cellular ATP Production Rate Using Agilent Seahorse XF Technology**).

With these considerations, the rate of mitochondrial ATP production is calculated according to Equation 4:

mitoATP Production Rate (pmol ATP/min) = OCR_{ATP} (pmol O_2/min) * 2 (pmol O/pmol O_2) * P/O (pmol ATP/pmol O) **(Equation 4)**

Finally, the total cellular ATP Production Rate (**Equation 5**) is the sum of the glycolytic and mitochondrial ATP production rates:

ATP Production Rate (pmol ATP/min) = glycoATP Production Rate (pmol ATP/min) + mitoATP Production Rate (pmol ATP/min) **(Equation 5)**

Glossary

- **glycoATP Production Rate:** Rate of ATP production (expressed in pmol ATP/min) associated with the conversion of glucose to lactate in the glycolytic pathway.
- **mitoATP Production Rate:** Rate of ATP production (expressed in pmol ATP/min) associated with oxidative phosphorylation in the mitochondria.
- **Total ATP Production Rate:** The sum of mitochondrial ATP and glycolytic ATP production rates in live cells under appropriate assay conditions.
- **P/O ratio:** number of molecules of ATP synthesized (that is, moles of ADP phosphorylated to ATP) per atom of oxygen (O) reduced by an electron pair flowing through the mitochondrial electron transport chain.
- **XF ATP Rate Index:** ratio of the mitoATP Production Rate divided by glycoATP Production Rate at a certain time point. This is a valuable metric for detecting changes and/or differences in metabolic phenotype. An increase in the XF ATP Rate Index represents a more oxidative / less glycolytic phenotype and vice-versa.
- **Induced Assay:** An XF Real-Time ATP Rate Assay workflow that includes an acute injection of an experimental compound prior to serial injections of oligomycin and rotenone/antimycin A. This workflow allows for measurement of *in situ* activation or repression of ATP production, as well as real-time shifts in the XF ATP Rate Index (mitoATP Production Rate / glycoATP Production Rate).
- **Buffer Factor:** Buffer capacity of the measurement system, comprising the assay medium and XF assay conditions (instrument, sensor, labware)
- **Glycolysis:** In the context of Seahorse XF Assays, the process of converting glucose to lactate.
- **Proton Efflux Rate:** The number of protons exported by cells into the assay medium over time, expressed as pmol/min.
- **Glycolytic Proton Efflux Rate:** 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.
- **ATP coupled respiration:** The portion of basal respiration that is being used to drive ATP production. It is quantified by injection of the ATP synthase inhibitor oligomycin.

2 Kit Information

Kit Contents

The Seahorse XF Real-Time ATP Rate Assay Kit includes six foil pouches, each containing reagents sufficient for one complete XF Real-Time ATP Rate Assay in either a 96 or 24-well Agilent Seahorse XF Cell Culture Microplate.

Every pouch includes one tube of Oligomycin and one tube of a mixture of Rotenone + Antimycin A (see **Table 1**).

Table 1 Seahorse XF Real-Time ATP Rate Assay Kit Contents

Compound	Target	Cap Color	Quantity per tube
Oligomycin	ATP synthase inhibitor (complex V)	Blue	63 nmol
Rotenone + Antimycin A (Rot/AA)	Mitochondrial ETC complexes I and III, respectively	Red	27 nmol each

Kit Shipping and Storage

Product ships at ambient temperature and can be stored at room temperature. Products are stable for 18 months from the date of manufacture (listed on the box).

Additional Agilent Products Required

The following Agilent products are also required for performing the Seahorse XF Real-Time ATP Rate Assay, but not supplied with the kit. For a complete list of materials required to perform an XF assay, please visit Basic Procedures to Run an XF Assay at
https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay

Item	Vendor	Part number
Seahorse XF Pro, XFe96 or XFe24 Analyzer	Agilent Technologies	
For Seahorse XF Pro or XFe96 analyzer:		
Seahorse XFe96/XF Pro FluxPak mini	Agilent Technologies	103793-100
or		
Seahorse XFe96/XF Pro FluxPak	Agilent Technologies	103792-100
For Seahorse XFe24 analyzer:		
Seahorse XFe24 FluxPak mini	Agilent Technologies	102342-100
or		
Seahorse XFe24 FluxPak	Agilent Technologies	102340-100
Seahorse XF DMEM Medium, pH 7.4	Agilent Technologies	103575-100
or		
Seahorse XF RPMI Medium, pH 7.4	Agilent Technologies	103576-100
Seahorse XF 1.0 M Glucose solution	Agilent Technologies	103577-100
Seahorse XF 100 mM Sodium Pyruvate solution	Agilent Technologies	103578-100
Seahorse XF 200 mM L-Glutamine solution	Agilent Technologies	103579-100

Narrow p1000 pipette tips are recommended for reconstituting compounds within the tube provided (for example, Fisherbrand SureOne Micropoint Pipet Tips, p/n 02-707-402).

3 Assay Workflow

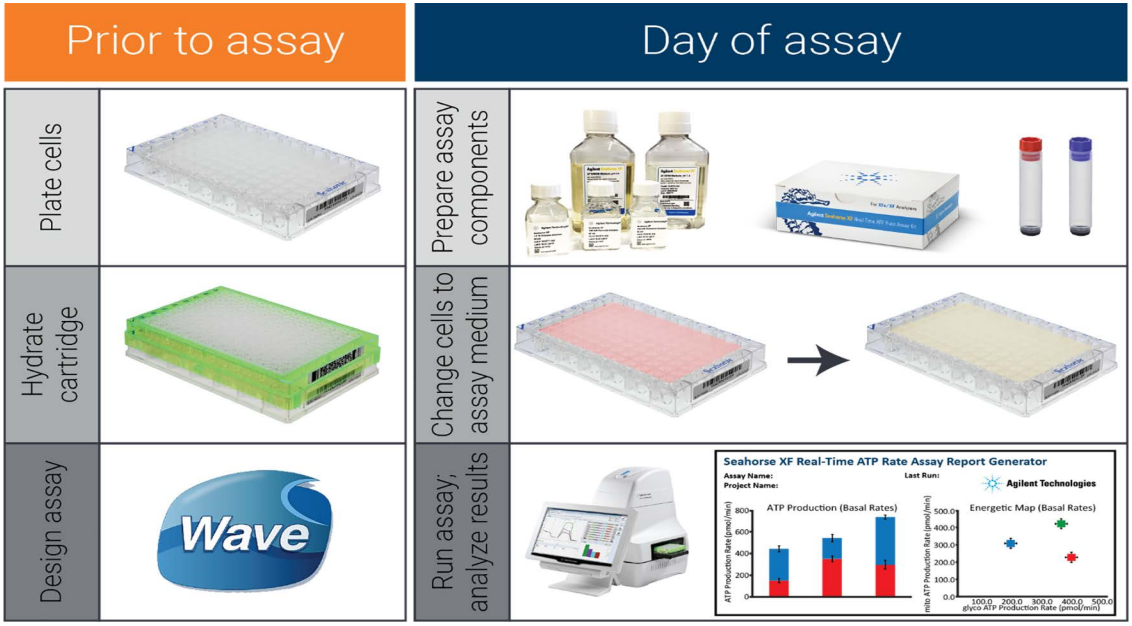

Figure 2. Agilent Seahorse XF Real-Time ATP Rate Assay workflow

Day Prior to Assay

1 Turn on the Seahorse XF Pro, XFe96 or XFe24 Analyzer and allow temperature to stabilize overnight.

2 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 procedures described in the Agilent Cell Analysis Learning Center: **https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay**.

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

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.

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

3 Hydrate a sensor cartridge at 37 ^{O}C in a non-CO_2 incubator overnight. (Refer to Basic Procedures in the Agilent Cell Analysis Learning Center).

4 Using the ATP Production Rate Assay template, design experiment in Wave Pro, Wave or Seahorse Analytics. Make any necessary group modifications to the template for the specific assay design.

Day of Assay

Prepare assay medium

1 Under sterile conditions, prepare assay media by supplementing 100 mL of Seahorse XF DMEM Medium, pH 7.4 with 10 mM of XF glucose, 1 mM of XF pyruvate, 2 mM of XF glutamine. These are recommended initial conditions; however, the desired assay medium composition can be varied depending on cell type or *in vitro* culture conditions.

2 Warm assay medium to 37 °C.

3 Incubate at 37 °C until ready to use.

NOTE

If the assay medium is significantly altered from this formulation (that is, higher concentrations of XF glucose, XF pyruvate or XF glutamine and/or other media additives), the pH of the assay medium should be checked and adjusted to 7.4 if needed, and the Buffer Factor Protocol must be performed to derive the buffer factor value. Consult Seahorse XF Buffer Factor Protocol User Guide.

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 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, remove the assay medium AGAIN and add fresh, warm assay medium to each well (see **Table 4** on page 18 for appropriate starting well volumes).

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 (XF Pro/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 match the appropriate Starting Well Volume as indicated in **Table 4** on page 18.

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

Prepare stock compounds

NOTE **Use compounds the same day they are reconstituted. Do not freeze. Discard any remaining compound.**

1 Remove one foil pack from the kit box, then open pouch and remove the Oligomycin (blue cap) and Rotenone/Antimycin A (red cap) vial.

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

3 Resuspend each component with the appropriate volume of prepared assay medium as described in **Table 2**. Vortex ~one minute to ensure full resuspension of compounds.

Table 2 Stock solutions

Compound	Volume of assay medium	Resulting stock concentration
Oligomycin	420 µL	150 µM
Rotenone + Antimycin A	540 µL	50 µM

Prepare compounds for loading in sensor cartridge

Prepare 3 mL of each compound in assay medium as described in **Table 3**. It is recommended to use 1.5 µM of oligomycin and 0.5 µM Rotenone + Antimycin A (final concentration).

Table 3 Compound preparation for performing the XF Real-Time ATP Rate Assay on an XF Pro, XFe96 or XFe24 Analyzer

	Stock volume (µL)	Medium volume (µL)	10X [Port] (µM)	[Final well] (µM)
Port A Oligomycin	300	2700	15	1.5
Port B Rot/AA	300	2700	5	0.5

Load sensor cartridge

- **Standard Assay** - No acute injection before oligomycin and rotenone/antimycin A. Load compounds into the following ports of a hydrated sensor cartridge:

  Port A: Oligomycin

  Port B: Rotenone/antimycin A

- **Induced Assay** -To inject a test compound(s), use port(s) A for the desired compound(s) and then load ports as follows:

  Port A: experimental compound (acute injection) or vehicle control

  Port B: Oligomycin

  Port C: Rotenone/antimycin A

Table 4 on page 18 lists the appropriate volumes and concentrations for injection schemes using two or more ports.

Table 4 Starting well assay medium and compound injection volumes.

	Agilent Seahorse XF Pro/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	75 µL	10X

Running the XF Real-Time ATP Rate Assay

1 Select the **Seahorse XF Real-Time ATP Rate Assay or Seahorse XF Real-Time ATP Rate Assay (Induced Assay)** 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 Map for your assay.

4 **Protocol**: Confirm or modify the Instrument Protocol for additional measurements cycles during the assay.

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

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

NOTE **Remove the cartridge lid and verify correct plate orientation.**

7 After completing the Calibration, the Wave Controller will display a Load Cell Plate message. Click **Open Tray** to eject the 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

Seahorse Analytics is a web-based software platform that provides a simple, streamlined data analysis workflow for analyzing XF Real-Time ATP Rate assay data.

Follow the steps below to use the relevant assay widgets in Seahorse Analytics:

1. After the assay is completed, transfer your assay result file to your office or personal computer using a USB drive or network drive.
2. To register or log in to your Seahorse Analytics account, visit: **https://seahorseanalytics.agilent.com**
3. Import the assay result file to your account and open the file.
4. Select Add View. Under Assay Kit Companion Views, click XF ATP Rate Assay, XF ATP Rate Screening, or XF ATP Rate Dose.
5. Select the desired analysis view(s), then click Add View.

For additional information about using Seahorse Analytics, visit the **Agilent Cell Analysis Learning Center** website.

4 Frequently Asked Questions

How is the mitoATP Production Rate calculated if there are different oxidizable substrates (with different P/O ratios) in the assay media?

To convert ATP-coupled respiration (OCR) into mitochondrial ATP production rates, the stoichiometry of ADP phosphorylated to ATP per atom of oxygen reduced at the terminal point of electronic transport chain must be determined. The maximum theoretical P/O value varies between different fuels and depends on the stoichiometry/pathway of the fuel oxidation, as well as the efficiency of the F_1F_0 ATP synthase. Under standard XF experimental conditions, cells are provided with a mixture of fuels (predominantly glucose, pyruvate and glutamine), and often endogenous fuel stores (glycogen, fatty acids, other amino acids) are available for mitochondrial oxidation. Thus, it was tested with several cell lines and validated that a P/O value of 2.75 accurately represents the average P/O ratio of the mixture of exogenous and endogenous oxidizable substrates. For more information, see the white paper: "Quantifying Cellular ATP Production Rate Using Agilent Seahorse XF Technology" at
https://www.agilent.com/cs/library/whitepaper/public/whitepaper-quantify-atp-production-rate-cell-analysis-5991-9303en-agilent.pdf

What is the XF ATP Rate Index as defined the in XF Real-Time ATP Rate Assay?

The XF ATP Rate Index is the ratio of the mitoATP Production Rate to the glycoATP Production Rate (i.e. mitoATP rate / glycoATP rate). This ratio represents a quantitative metric of the cellular metabolic phenotype. Metabolic indices greater than 1.0 represent cellular metabolism where greater than 50 % of cellular ATP is derived from mitochondrial through ETC/OXPHOS, while indices less than 1 indicate greater than 50 % of total ATP is produced by the glycolytic pathway. Since the metabolic index is a ratio-metric measurement, it is highly sensitive to changes or shifts in metabolic phenotype.

Why is the XF ATP Rate different for the same cell type when the cells are plated at different densities?

The metabolic phenotype of the cell will be influenced by the cellular demand for ATP. Cells that are proliferating or differentiating have, in general, higher glycolytic rates than cells that are confluent (slow growing) or terminally differentiated. In order to compare results between experiments it is recommended to maintain consistent cell culture conditions and cell seeding densities throughout the investigation.

Why must Seahorse XF DMEM Medium, pH 7.4 or Seahorse XF RPMI Medium, pH 7.4 be used to perform the assay?

The calculation of the glycoATP Production Rate requires an absolute measurement of the glycolytic proton efflux rate during the XF Real-Time ATP Rate Assay. To properly calculate PER, the assay media must have a fixed buffer capacity. A low concentration of HEPES in the media provides consistent buffer capacity values across the time frame of the assay. Although addition of HEPES buffer reduces the ECAR signal slightly, it significantly improves the quality and consistency of the ECAR data, as well as the accuracy of the transformation to PER (For more details see **White paper: Improving Quantification of Cellular Glycolytic Rate Using Agilent Seahorse XF Technology**. Additionally, the use of XF DMEM or XF RPMI medium with the pH pre-adjusted to 7.4 saves time in assay preparation and ensures a consistent assay media pH value across XF experiments.

Can I use other Seahorse XF assay media to run the XF Real-Time ATP Rate Assay?

Calculation of accurate glycoATP production rates requires the use of an assay medium without phenol red or bicarbonate and a low concentration of HEPES buffer. Thus, the use of Agilent Seahorse XF DMEM (or RPMI), pH 7.4 is strongly recommended. Any deviation from the recommended medium and supplements (glucose, pyruvate, glutamine) will require that the Buffer Factor should be determined empirically (using the XF Analyzer) for each assay (See the Seahorse XF Buffer Factor Protocol for further information). Any assay medium containing phenol red cannot be used for the XF Real-Time ATP Rate Assay.

When running an induced XF Real-Time ATP Rate Assay, the OCR after oligomycin injection is higher than the basal OCR, what happened?

Addition of compounds that uncouple electron transport from oxidative phosphorylation (like FCCP, DNP, etc.) before the oligomycin injection will typically result in an increase in OCR. However, this respiration is not coupled to ATP production since the mitochondrial membrane potential is decreased or dissipated without participation of the ATP synthase, so little to no ATP is generated under these circumstances. In these cases, the basal mitoATP Production Rate cannot be calculated accurately. If an uncoupler compound is suspected, then it is recommended to include a control group with an injection of assay medium + vehicle to calculate basal mitoATP Production Rates.

Can I perform this assay with Spheroids, organoids, or tissue materials?

No. This assay and the parameters of glycoATP and MitoATP production rates are not validated for 3-dimensional biological models. This assay is not compatible with XFe96 Spheroid or XF24 Islet plates.

4 Frequently Asked Questions

www.agilent.com

DE-003334

© Agilent Technologies, Inc. 2024

Second edition, December 2024

103592-400 Rev C