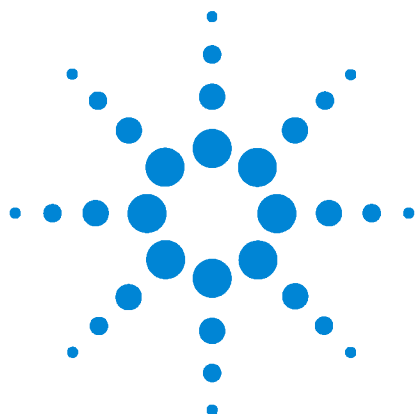




Agilent Seahorse XF Glycolytic Rate Assay Kit

**User Guide
Kit 103344-100**



Agilent Technologies

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

103344-400

Kit Part Number

103344-100

Edition

Third edition, December 2024
Revision F

Printed in USA

Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610 USA

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

If software is for use in the performance of a U.S. Government prime contract or subcontract, Software is delivered and licensed as “Commercial computer software” as defined in DFAR 252.227-7014 (June 1995), or as a “commercial item” as defined in FAR 2.101(a) or as “Restricted computer software” as defined in FAR 52.227-19 (June 1987) or any equivalent agency regulation or contract clause. Use, duplication or disclosure of Software is subject to Agilent Technologies’ standard commercial license terms, and non-DOD Departments and Agencies of the U.S. Government will receive no greater than Restricted Rights as defined in FAR 52.227-19(c)(1-2) (June 1987). U.S. Gov-

ernment users will receive no greater than Limited Rights as defined in FAR 52.227-14 (June 1987) or DFAR 252.227-7015 (b)(2) (November 1995), as applicable in any technical data.

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

Introduction

Assay Background 5

Glossary 9

Kit Information

Kit Contents 11

Kit Shipping and Storage 11

Additional Required Items 12

Assay Workflow

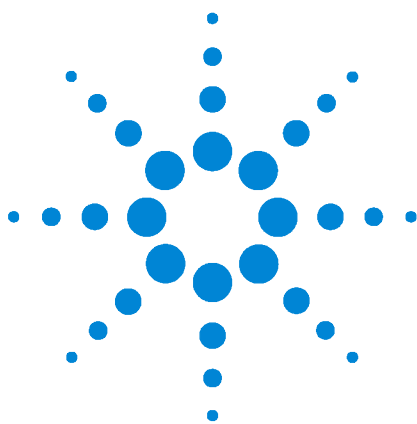
Day Prior to Assay 14

Day of Assay 14

Running the Assay 18

Data Analysis 18

Frequently Asked Questions



1

Introduction

Assay Background 5

Glossary 9

Assay Background

For use with Agilent Seahorse XF Pro, 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, XF Pro 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 CO₂ to extracellular acidification derived from mitochondrial/TCA cycle activity and are directly comparable to lactate accumulation data.



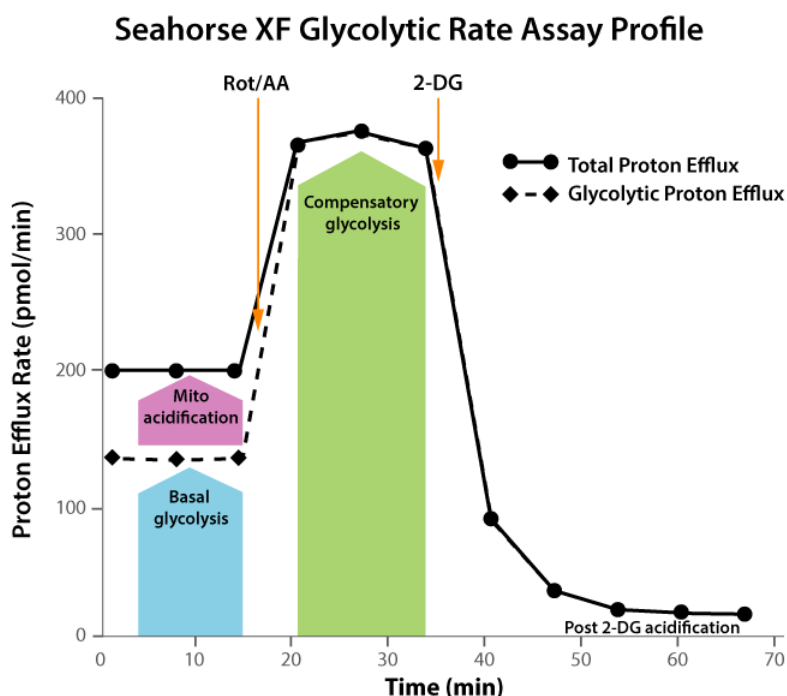


Figure 1 Agilent Seahorse XF Glycolytic rate assay profile. Proton efflux from live cells comprises both glycolytic and mitochondrial-derived acidification. Inhibition of mitochondrial function by Rotenone and Antimycin A (Rot/AA) enables calculation of mitochondrial-associated acidification. Subtraction 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 CO₂ 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/CO₂ to extracellular acidification is calculated and used to subtract the CO₂ contributing acidification from the total Proton Efflux Rate (PER). (For a detailed explanation, please read the *Agilent Seahorse XF CO₂ 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 CO₂- 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.

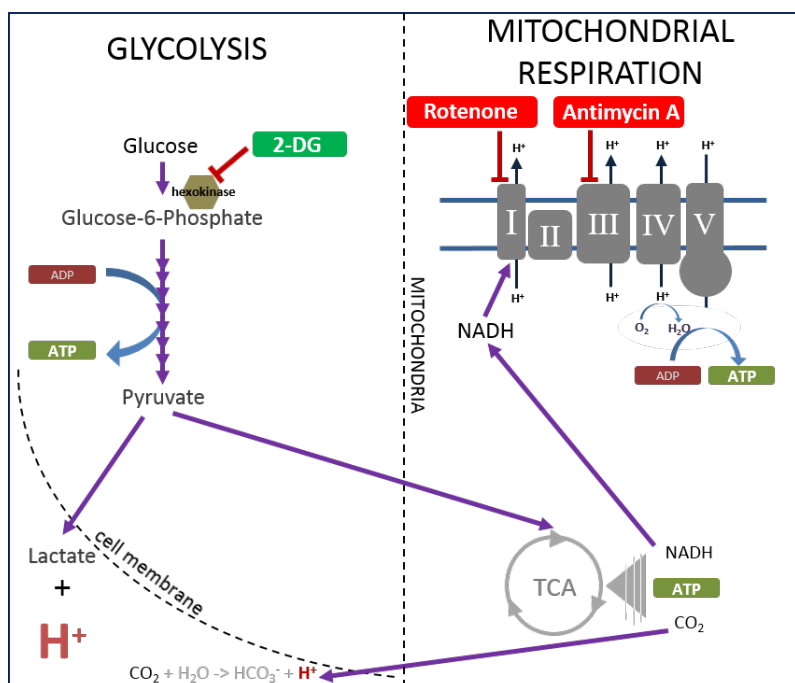
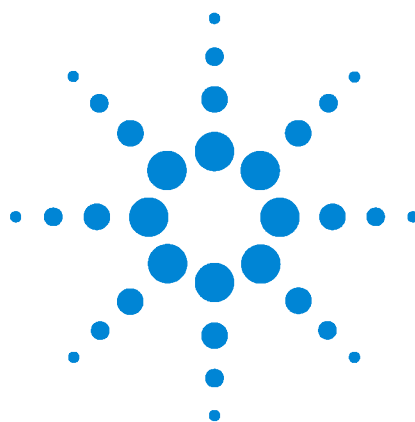


Figure 2 Principle of the Agilent Seahorse XF Glycolytic rate assay. Energy is produced by two pathways in the cell - glycolysis and mitochondrial respiration. In the breakdown of glucose to lactate during glycolysis, protons are extruded into the extracellular media which is detected by the XF Analyzer as ECAR. Additionally, mitochondrial TCA activity produces CO₂, 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 inhibitor of glycolysis is injected to stop glycolytic acidification.

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 CO₂-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 [11](#)
Kit Shipping and Storage [11](#)
Additional Required Items [12](#)

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 μ mol

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

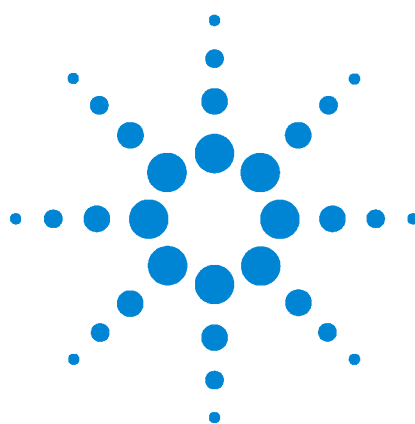
The following items are required to run the Seahorse XF Glycolytic Rate Assays, but not supplied with the kit.

Items	Supplier	Catalog number
Agilent Seahorse XF Analyzers	Agilent Technologies	
For XF Pro or XFe96 Analyzers: Seahorse XF Pro M FluxPak (XF Pro only)	Agilent Technologies	103777-100 or 103775-100 103792-100 or
Seahorse XFe96/XF Pro FluxPak	Agilent Technologies	103793-100 or 103798-100
For XFe24 Analyzers: Seahorse XFe24 FluxPak	Agilent Technologies	102340-100 or 102342-100
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

Day Prior to Assay 14
Day of Assay 14
Running the Assay 18
Data Analysis 18

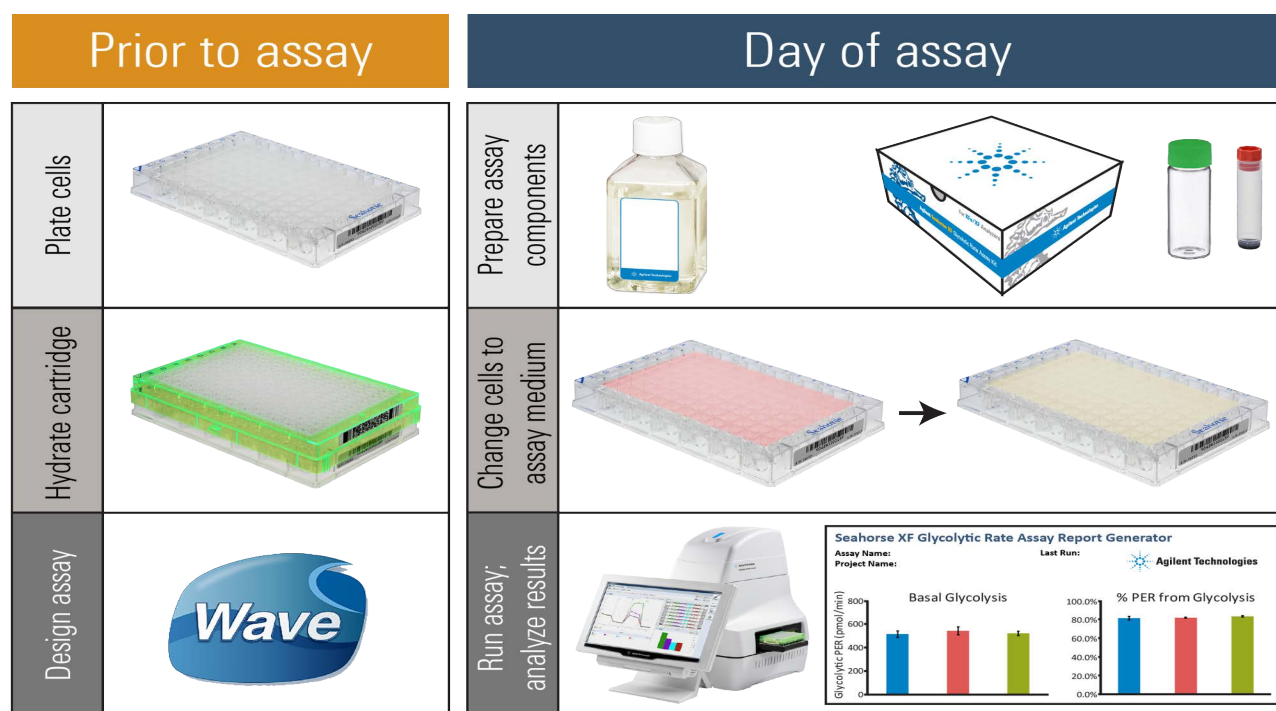


Figure 3 Agilent Seahorse XF Glycolytic Rate Assay Workflow



Day Prior to Assay

- 1 Turn on the Seahorse XF Analyzer, and allow the temperature to stabilize overnight.
- 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-CO₂ 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 CO₂ 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-CO₂ 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 (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 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-CO₂ 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 XF Pro, 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 XF Pro/XF96 Analyzer		Agilent Seahorse XFe24 Analyzer	
	Starting well volume: 180 μ L assay medium		Starting well volume: 500 μ L assay medium	
Port	Vol.	Conc.	Vol.	Conc.
A	20 μ L	10X	56 μ L	10X
B	22 μ L	10X	62 μ L	10X
C	25 μ L	10X	69 μ L	10X
D	27 μ L	10X	76 μ L	10X

Running the Assay

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.

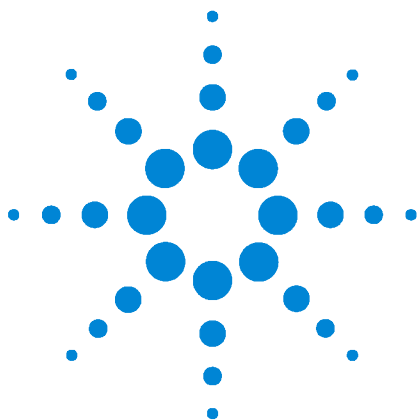
Data Analysis

Seahorse Analytics is a web-based software platform that provides a simple, streamlined data analysis workflow for analyzing Glycolytic 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 Glycolytic Rate Assay.
- 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

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 CO₂ effect the ECAR measurements?

Acidification of the media by CO₂ 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 CO₂ 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 CO₂.

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.

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

No. This assay is not validated for 3-dimensional biological models. This assay is not compatible with XFe96 Spheroid or XF24 Islet plates.



© Agilent Technologies, Inc.

Printed in USA, December 2024
Revision F

DE-003332



103344-400