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# Agilent Seahorse XF $CO_2$ Contribution Factor Protocol

## User Guide

Agilent Technologies

# Notices



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**CAUTION**

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# 1
# Introduction



## Assay Background

Measurements of extracellular acidification rate (ECAR) have been used to study glycolysis, assuming the extrusion of lactate constitutes the principal source of extracellular acidification. However, $CO_2$ production resulting from mitochondrial activity can also contribute to extracellular acidification. Most $CO_2$ produced in the cells is derived from the TCA cycle, which is a metabolic route tightly coupled to mitochondrial respiration. Mitochondrial-derived $CO_2$ can partially hydrate in the extracellular medium yielding $H^+$ + $HCO_3$, resulting in additional acidification of extracellular medium. The $CO_2$ Contribution Factor (CCF) is an empirically derived value that allows the conversion of mitochondrial respiration (mitoOCR) into $CO_2$-dependent proton efflux rate (PER) to discount $CO_2$ contribution to PER. The resulting value, Glycolytic Proton Efflux Rate (glycoPER), is the rate of protons extruded in the extracellular medium due specifically to glycolysis. Based on measurements of CCF across 20 cell lines in multiple Agilent Seahorse instrument models, constant values for CCF were determined to correlate mitochondrial respiration with $CO_2$ contribution to PER, as shown in Table 1.

**Table 1** Average $CO_2$ Contribution Factor (CCF) for different Agilent Seahorse instrument types

| Agilent Seahorse instrument type | CCF |
|---|---|
| XFp Analyzer | 0.51 ± 0.11 |
| XFe24 Analyzer | 0.60 ± 0.10 |
| XFe96 and XF96 Analyzers | 0.61 ± 0.13 |

## Procedure Overview

These values are entered as defaults in the Agilent Seahorse XF Glycolytic Rate Assay Report Generator, and can be used for most cell lines. However, the CCF value can be slightly different depending on mitochondrial fuels, cellular carbonic anhydrase content, and so forth. Accuracy of CCF for a particular cell model can impact the accuracy of the glycolytic rates, especially for cells that are highly oxidative. To improve the accuracy of Seahorse XF Glycolytic Rate Assay results for highly oxidative cells (% of acidification due to glycolysis obtained during Glycolytic Rate Assay is < 50%), we recommend that researchers confirm CCF value in their cellular model and assay conditions using the following protocol.

To calculate CCF, a Seahorse XF Cell Mito Stress Test is performed using glucose-free Glycolytic Rate Assay medium. The absence of glucose prevents glycolysis from occurring and causing acidification. In the absence of glucose, acidification is mostly due to $CO_2$ hydration/dissociation; thus OCR and ECAR profiles should exhibit the same pattern of responses to compound injections.

# 2
# Materials Required



## Equipment/software

- Agilent Seahorse XF96, XFe24, XFe96, or XFp Analyzer
- Non-$CO_2$ incubator
- Wave 2.3 (Desktop or XFe Controller) or higher
- Microsoft Excel for Windows or Mac

## Reagent/consumables

- Agilent Seahorse XF Base medium without Phenol Red (p/n 103335-100)
- 1M HEPES (p/n 103337-100)
- Agilent Seahorse XF Cell Mito Stress Test Kit (p/n 103015-100 for XF/XFe; p/n 103010-100 for XFp)
- Glutamine and pyruvate (same as for other Seahorse assays)
- Agilent Seahorse XF Assay Cartridge and Calibrant
- Agilent Seahorse XF Cell Culture Microplate or Miniplate

Agilent Seahorse products are available on the online store:
http://www.chem.agilent.com/store/

# 3
# Assay Workflow



## Day Prior to Assay

1 Turn on the Agilent Seahorse XF/XFe96 or XFe24 Analyzer, and let it warm up to stabilize.

2 Hydrate a sensor cartridge in Seahorse XF calibrant at 37 °C in a non-$CO_2$ incubator overnight.

3 For adherent cells, plate cells at a predetermined density in the Seahorse XF Cell Culture Microplate or Miniplate using the appropriate cell culture growth medium.
**NOTE**: For details on performing these procedures for specific instruments, please refer to the Basic Procedures section of agilent.com:
http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay

4 For suspension cells, see “Prepare Seahorse XF Cell Culture Microplate for assay” on page 10.

# Day of Assay

## Prepare assay medium

1. If using an XFp Analyzer, turn it on and select the **Cell Mito Stress Test Assay** template.
2. Prepare the assay medium identical to the one that you intend to use for the Seahorse XF Glycolytic Rate Assay but without glucose. Remember that assay medium has to be phenol red- and bicarbonate free and contain a low concentration of a buffer such as 5 mM HEPES.
3. Warm the assay medium to 37 °C.
4. Adjust the pH to 7.4 with 1 N NaOH

**NOTE** Agilent Seahorse recommends sterile filtration following pH adjustment.

5. Incubate at 37 °C until ready to use.

### Standard assay medium for CCF calculation

Seahorse XF base medium without phenol red + 5 mM HEPES + 2 mM glutamine + 1 mM pyruvate, pH7.4

**NOTE** If the assay medium is substantially changed from this formulation, the Buffer Factor Protocol User Guide must be used to derive the BF value.

## Prepare Seahorse XF Cell Culture Microplate for assay

### Adherent cells

1. Remove the cell culture microplate (or miniplate) from the 37 °C $CO_2$ incubator, and examine the cells under a microscope to confirm consistent plating and proper cell morphology.
2. Wash the cells. For more details, refer to the Basic Procedures section of agilent.com: http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay.

3 Remove the cell culture growth medium in the cell culture microplate (or miniplate). 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 60 minutes prior to the assay.

4 Before starting the XF assay, remove the assay medium, and add fresh, warm assay medium (see Table 2).

### Suspension cells

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

2 Count the cells and suspend at a concentration such that seeding 50 µL of cells (for XFp or XF96/XFe96) or 100 µL (for XFe24) contains the desired cell number per well, leaving 2-4 wells without cells as background correction wells.

3 Add 50 µL (XFp/XF96/XFe96)/100 µL (XFe24) cells/well, then centrifuge gently to adhere. Gently add the corresponding volume of assay medium to each well to obtain the starting assay medium volume indicated in Table 2 on page 11.

4 Incubate the plate in a 37 °C, non-$CO_2$ incubator for 60 minutes prior to the assay.

**Table 2** Medium volumes

| | Seahorse XFp/ XFe96/ XF96 Analyzer | Seahorse XFe24 Analyzer |
|---|---|---|
| Cell seeding volume | 50 µL | 100 µL |
| Starting assay volume (constant port volume) | 175 µL | 525 µL |
| Starting assay volume (constant port concentration) | 180 µL | 500 µL |

## Run an Agilent Seahorse XF Cell Mito Stress Test

Prepare compounds, load sensor cartridge, and run the assay following the Seahorse XF Cell Mito Stress Test User Guide for XF/XFe or XFp Analyzers.

# Post-Run Analysis

After completing the assay, follow the steps below to determine the CCF for your cell model using the Agilent Seahorse XF $CO_2$ Contribution Factor Calculator.

1. Using Wave 2.3 (or higher), open the assay result file (.asyr) to view result data.
2. Click the **Export** button, and select **Microsoft Excel**.
3. Choose a location to save the Excel file and click **Save**.
4. Download the Seahorse XF $CO_2$ Contribution Factor Calculator from the Report Generators page on agilent.com: http://www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-xf-report-generators.
5. Double-click the macro-enabled Excel (.xltm) file to open the Seahorse XF $CO_2$ Contribution Factor Calculator.
6. Click **Load New Data File**.
7. Locate the exported Excel file, and click **OK**.
8. Use the **Display Options** window to select groups to calculate the CCF (Figure 1).
9. Click **Update Summary** to calculate the custom $CO_2$ Contribution Factor for each group selected (see Figure 2 on page 13). Add the calculated CCF(s) to the Seahorse XF Glycolytic Rate Assay Report Generator (**Advance** tab) before creating the custom Summary Report.

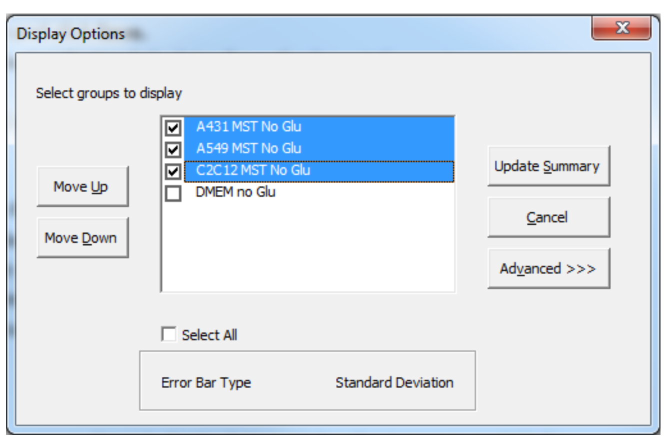


**Figure 1** Display Options window showing groups available for selection from the imported Excel file.

| Group | Value | StdDev |
|---|---|---|
| A431 MST No Glu | 0.63 | 0.06 |
| A549 MST No Glu | 0.70 | 0.14 |
| C2C12 MST No Glu | 0.55 | 0.02 |

**Figure 2** Table of the calculated $CO_2$ Contribution Factors displayed on the Summary Printout tab for the selected groups.

## Advanced options

Click the **Advanced** button to display and, if necessary, edit the default BF for each group selected (see Figure 3). The default BF value in the Seahorse XF $CO_2$ Contribution Factor Calculator is the BF for the recommended Glycolytic Rate Assay Medium. If a different assay medium is used, use the Seahorse XF Buffer Factor Calculator to calculate the custom BF. Enter the custom BF(s) in the Advanced options in the Seahorse $CO_2$ Contribution Factor Calculator then click **Update Summary** to apply the custom BF(s) to the table of calculated $CO_2$ Contribution Factor(s) (Figure 2).

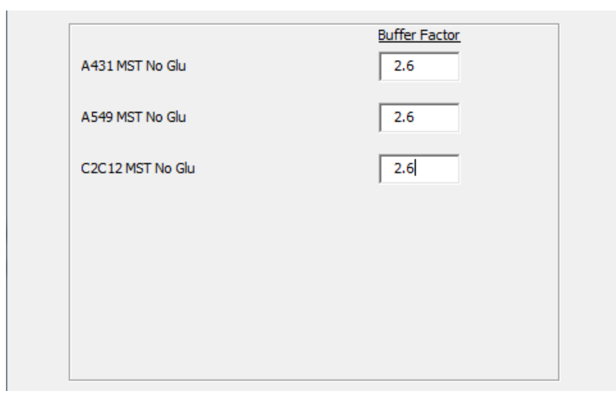


**Figure 3** Advanced options showing the selected groups and corresponding buffer factor value.

# 4
# Frequently Asked Questions

### What should I do if I see a warning message about an increase in ECAR after the oligomycin injection or a negative CCF value in my experiment?

The CCF protocol is based on the assumption that in the absence of glucose in the extracellular medium, and after 1 hour of glucose starvation, glycolysis-dependent acidification is fully inhibited and all observed acidification is due to $CO_2$ production. However, some cells have Glycogen stores that are hydrolyzed and fuel Glycolysis, in particular after inhibiting mitochondrial ATP production with oligomycin or Rotenone and Antimycin A (Rot/AA). For these cell lines, CCF cannot be calculated using the Seahorse XF $CO_2$ Contribution Factor Protocol; using the average validated value is recommended for Glycolytic Rate Assay calculations.

### Can I use this assay to determine the $CO_2$ production rate of my cells?

No- this assay measures only the amount of $CO_2$ that acidifies the media, which is less than the amount of $CO_2$ produced. The measured proton efflux rate is influenced by cellular factors as well as features of the measurement system (see Table 1 on page 5); therefore, it cannot be used to determine actual $CO_2$ production by the mitochondria. Because $O_2$ consumption is so tightly coupled to $CO_2$ production, mitochondrial $O_2$ consumption is the better indicator of mitochondrial $CO_2$ production.

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