



Agilent Seahorse XF Buffer Factor Protocol

## Quick Reference Guide



# Notices

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# 1

# Introduction

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## Assay Background

In Agilent Seahorse XF Analyzers, extracellular acidification rate (ECAR) provides a qualitative measurement of proton extrusion in the extracellular medium. This rate is reported as milli-pH/minute (mpH/min); as such, the values are highly dependent on the buffer capacity of the medium and the properties of the measurement chamber. Determining and reporting the actual amount of protons being exported from the cell over time requires that we know the Buffer Factor (BF) of the measurement system. Unlike buffer capacity, which is an inherent property of a particular solution, BF is a function of the buffer capacity of the media as well as the system components (for example, instrument, sensor material, labware). BF is defined as the amount of  $H^+$  added to the assay medium to change the pH level by 1 pH unit as measured in an Agilent Seahorse XF instrument, and expressed as mM/pH. Once the BF for a measurement system is known, the qualitative ECAR can be converted into a quantitative Proton Efflux Rate (PER).

## Procedure Overview

BF is determined empirically within the XF instrument by titrating (via port injection) known amounts of acid to the assay medium, and measuring the pH value after each addition.

The protocol involves preparing an assay plate (8-16 wells) with the assay medium to be measured following an analogue procedure as a typical assay, but without cells. Buffer factor(s) for up to two different assay media can be analyzed at the same time with the Agilent Seahorse XF Buffer Factor Calculator.

The cartridge ports are loaded with 5.0 mM HCl solution prepared in assay medium, which is then injected during the course of the assay. Wells with medium-only injections are included as a control.

The degree to which the pH drops after each injection of a known amount of acid determines the BF. The Seahorse XF Buffer Factor Calculator performs these calculations, and reports the BF as the amount of H<sup>+</sup> added to the assay media to change pH level 1 unit measured in a Seahorse XF instrument, and is expressed as mM/pH.

## 1 Introduction

(A)

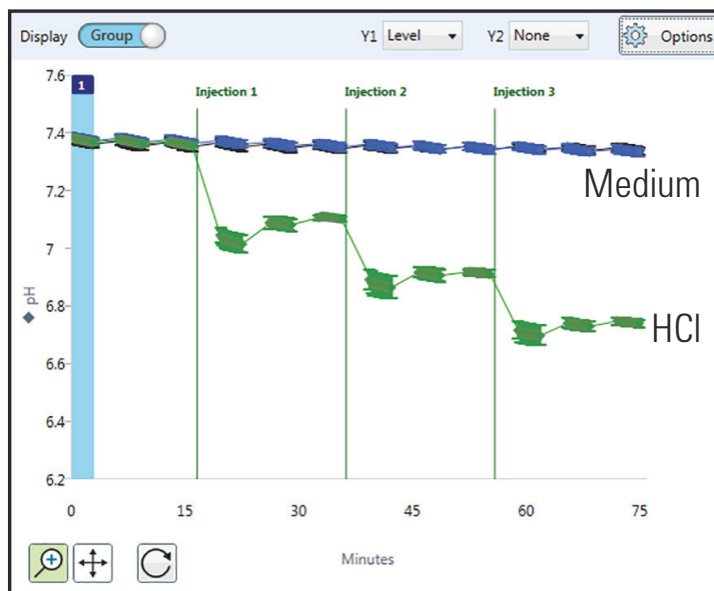


Figure 1. Calculation of BF of assay medium.  
(A) Titration of acid into assay medium using port injection as shown in the Wave software. Green: injection of 5.0 mM HCl in assay medium. Blue = injection of assay medium as control.  
(B) Calculation of BF from pH levels after each injection as shown in (A).



## 1 Introduction

(B)

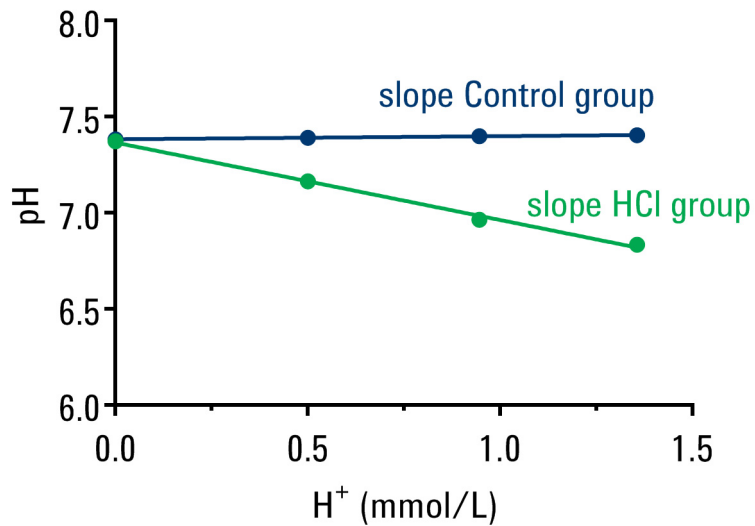


Figure 1. Calculation of BF of assay medium.  
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(B) Calculation of BF from pH levels after each injection as shown in (A).

## 1 Introduction

## 2

# Materials Required

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# Equipment/Software

- Agilent Seahorse XFp, XF96, XFe24, or XFe96 Analyzer
- Non-CO<sub>2</sub> incubator
- 10 mL volumetric flask
- Wave 2.4 (Desktop or Controller) or higher required.
- Wave Desktop 2.6 recommended:  
<https://www.agilent.com/en/products/cell-analysis/software-download-for-wave-desktop>
- Microsoft Excel for Windows or Mac

# Reagent/Consumables

- Assay medium desired to run the Seahorse XF Real-Time ATP Rate Assay. Like other assay media to be used in XF assays that require measurement of PER, the assay medium has to be bicarbonate free, phenol red free and contain a low concentration of a buffer system like 5 mM HEPES. For more details see Seahorse XF Glycolytic Rate Assay Kit User Guide, <https://www.agilent.com/cs/library/usermanuals/public/103344-400.pdf>.
- Agilent Seahorse XF or XFp FluxPack
- HCl 0.1 N volumetric hydrochloric acid solution (such as J.T. Baker 562102)
- Cell culture growth medium prepared as regularly for the cell type of interest

## 2 Materials Required

# 3

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## Day Prior to Assay

- 1 Turn on the Seahorse XF/XFe/XFp Analyzer, and let it warm up to stabilize.
- 2 Hydrate a sensor cartridge in distilled or deionized sterile water at 37 °C in a non-CO<sub>2</sub> incubator overnight. 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):  
[http://www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay)
- 3 Prepare a Seahorse XF cell culture microplate or XFp cell culture miniplate as you would for a regular cell-based assay.
  - For BF calculation of assay medium to be used in assays with adherent cells:
    - a Add 80 µL (for XF96/XFp plates) or 250 µL (for XFe24 plates) of cell culture growth medium to the wells of an XFp cell culture miniplate/XF96 or XF24 cell culture microplate to be used for the BF calculation (recommend 8-16 wells per plate).
    - b Incubate the plate in a CO<sub>2</sub> incubator overnight (analogous to cell culture conditions for a Seahorse XF Assay or can be some empty wells of your cellular assay).
      - For BF calculation of assay medium to be used for suspension cell assays, see **“Day of Assay”** on page 17.
- 4 Select the XF Buffer Factor Assay template (in Wave 2.4 or higher) and make any necessary edits before starting the assay.

Alternatively, you can create your own Buffer Factor assay template following the steps below:

- a Define a Control and HCl group.
- b Assign at least 4-8 wells to each group. Assay “background wells” can be used as control wells for this method if assay medium is injected through port injection.
- c Ensure the Instrument Protocol has at least three baseline measurement cycles, three injections, and three measurement cycles following each injection command.

### NOTE

**In order to calculate a custom BF using the Seahorse XF Buffer Factor Calculator, the assay must have three injections.**



## Day of Assay

### Prepare assay medium

- 1 Prepare assay medium identical to the one to be used for the XF Real-Time ATP Rate Assay. Remember that assay medium has to be phenol red- and bicarbonate free and contain low concentration of a buffer system such as 5 mM HEPES.
- 2 Warm the assay medium to 37 °C.
- 3 Adjust the pH to 7.4 with 1 N NaOH. Keep at 37 °C until ready to use.

#### NOTE

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

### Prepare assay plate

- 4 Add assay medium.
  - For Adherent cell experiments, remove the cell culture medium from the Seahorse cell culture plate, and replace it with “Initial Well Volume” of prewarmed assay medium to all wells. (See **Table 1** on page 18.)
  - For Suspension cell experiments, add “Initial Well Volume” of prewarmed assay medium to an empty XF cell culture plate to wells corresponding to background, control group and HCl group.
- 5 Incubate approximately 45-60 minutes at 37 °C in a non-CO<sub>2</sub> incubator. Keep the assay media bottle at 37 °C.

### Prepare cartridge

- 6 Create HCl working solution: Using a volumetric flask, dilute 500 µL of volumetric 0.1 N HCl solution in 9.50 mL of assay medium to yield 5.0 mM HCl in assay medium.
- 7 Load the sensor cartridge with assay medium (control or background wells) or HCl Working Solution (HCl wells) in the chosen wells using the volumes in **Table 1** on page 18.

**Table 1 Well and Port Loading Volumes for the BF Protocol.**

Loading volumes	Agilent Seahorse XF96, XFe96, and XFp analyzers	Agilent Seahorse XFe24 analyzer
Initial well volume	175 $\mu$ L	525 $\mu$ L
Ports A, B, and C	25 $\mu$ L	75 $\mu$ L

## Final assay plate wash

- 8 Replace the assay medium in the cell culture microplate with prewarmed assay medium using the “Initial well volume” in **Table 1**.
- 9 Run the XF assay. Use the appropriate assay template as described in **“Day Prior to Assay”** on page 16.

## Post-Run Analysis Using the Agilent Seahorse XF Buffer Factor Calculator

After completing the assay, follow the steps below to determine the BF of your assay medium using the Seahorse XF Buffer Factor Calculator.

- 1 Using Wave Desktop 2.6, open the assay result file (.asyr) to view result data.
- 2 Open the **Overview** tab.
- 3 Use the **Group List** to select the HCl Injection and Control Injection groups to display on the kinetic graph.
- 4 Use the **Rate** drop-down menu to select ECAR data.
- 5 Use the **Y1** drop-down menu to select Level data.
- 6 Verify the initial pH level of the select wells is in the range  $7.4 \pm 0.2$ . (See **Figure 1** on page 8.)
- 7 Click the **Export** button and select **Microsoft Excel**.
- 8 Choose a location to save the Excel file and click **Save**.
- 9 Download the Seahorse XF Buffer Factor Calculator from the Report Generators page on agilent.com.
- 10 Double-click the macro-enabled Excel (.xlsm) file to open the Seahorse XF Buffer Factor Calculator.
- 11 Click **Load New Data File**.
- 12 Locate the exported Excel file and click **OK**.
- 13 If only 1 assay medium is used, use the **1st Set** drop-down menus displayed on the **Load Options** window to assign the HCl injection Group and the Control Injection Group (assay medium injections). (See **Figure 2** on page 20.)

### 3 Assay Workflow

If a second assay medium has been tested, use the **2nd Set** drop-down menus to assign the HCl Injection group and the Control Injection group (assay medium injections).

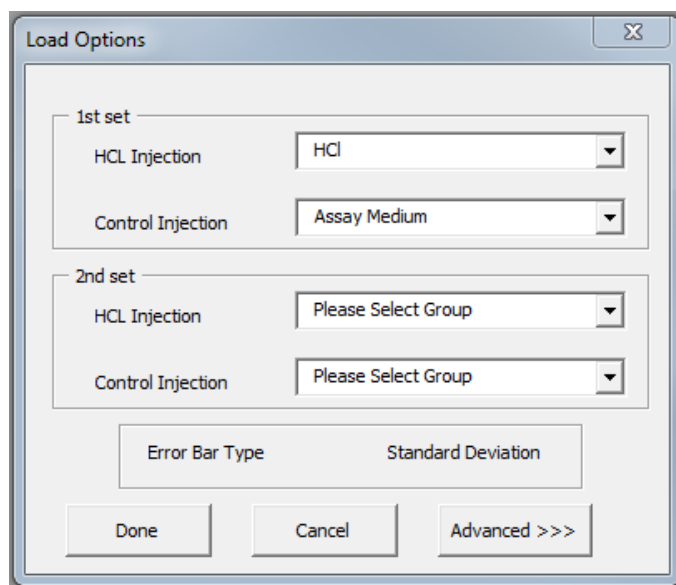


Figure 2. Assign the HCl and Control Injection groups using the Load Options window.

- 14 Click **Done** to calculate the custom BF of each assay medium. (See **Figure 3**.)
- 15 Go back to your XF Real-Time ATP Rate assay result file in Wave Desktop 2.6, and add the calculated BF value to the Assay Media group definition as well as the background wells before exporting result data to Microsoft Excel for analysis using the Seahorse XF Real-Time ATP Rate Assay Report Generator. For step-by-step instruction, see the Quick Reference Guide: Calculating Proton Efflux Rate (PER) Data, [https://www.agilent.com/cs/library/quickreference/public/Proton\\_Efflux\\_Rate\\_Quick\\_Reference\\_Guide\\_Final.pdf](https://www.agilent.com/cs/library/quickreference/public/Proton_Efflux_Rate_Quick_Reference_Guide_Final.pdf)

### 3 Assay Workflow

#### Buffer Factor

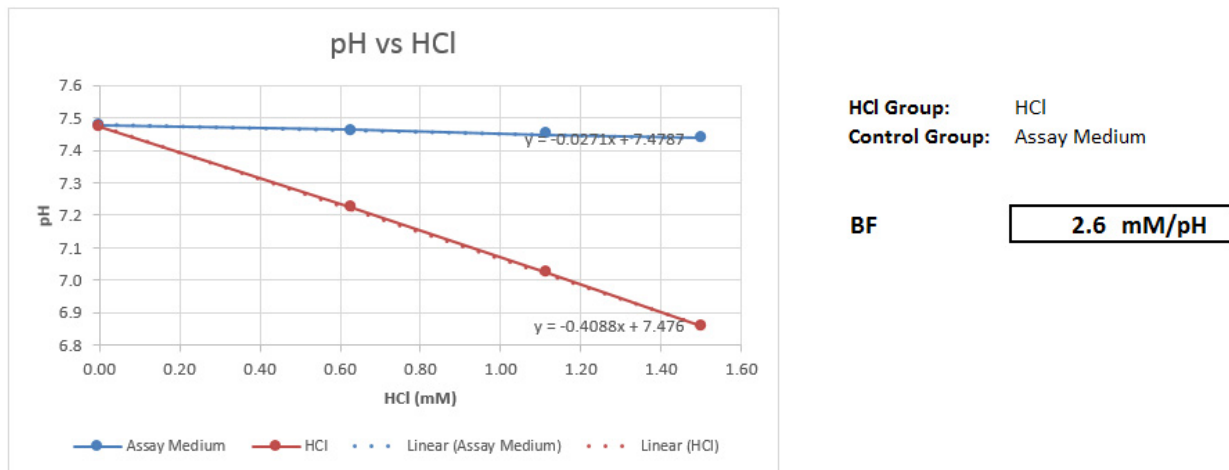
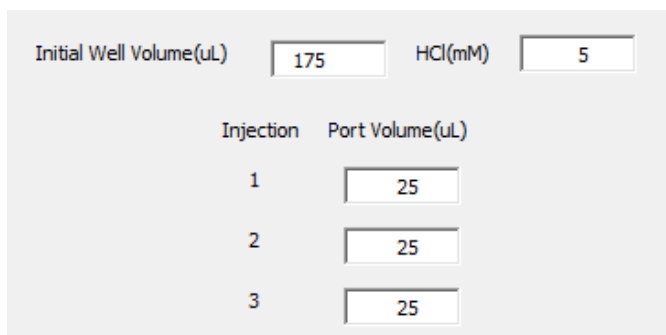


Figure 3. Example Summary Report displaying the custom BF calculated for the HCl group.

## Advanced Options

Click the **Advanced** button to display the default values for Initial Well Volume ( $\mu\text{L}$ ), HCl Stock Concentration (mM), and Port Volume ( $\mu\text{L}$ ) for each injection. (See **Figure 4**.) For assays using the same concentration and volumes outlined in the Seahorse XF Buffer Factor Protocol User Guide, do not modify the default values under Advanced options. For assays using a concentration or volume that is different than the specified default value, enter the custom value(s) before loading data in the Buffer Factor Calculator. Once finished, click **Done** to display the calculated BF.



Initial Well Volume( $\mu\text{L}$ )	<input type="text" value="175"/>	HCl(mM)	<input type="text" value="5"/>
Injection	Port Volume( $\mu\text{L}$ )		
1	<input type="text" value="25"/>		
2	<input type="text" value="25"/>		
3	<input type="text" value="25"/>		

Figure 4. Advanced options in the Seahorse XF Buffer Factor Calculator Report Generator.

**NOTE**

The graph in the Seahorse XF Buffer Factor Calculator represents the pH values measured in the well after each HCl or medium injection. The slope of the HCl Group line (**Figure 3** on page 21) has to be negative and significantly different from the Control Group line.

**NOTE**

To run the XF Real-Time ATP Rate Assay, the BF of your assay medium should have a value in the range of 1.8 - 4.0 mM/pH. Values outside the range will result in deviations in calculations and inaccurate glycolytic rate results.



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