

# Report Generator User Guide

Agilent Seahorse  
XF Cell Mito Stress Test  
&  
Agilent Seahorse  
XF Glycolysis Stress Test

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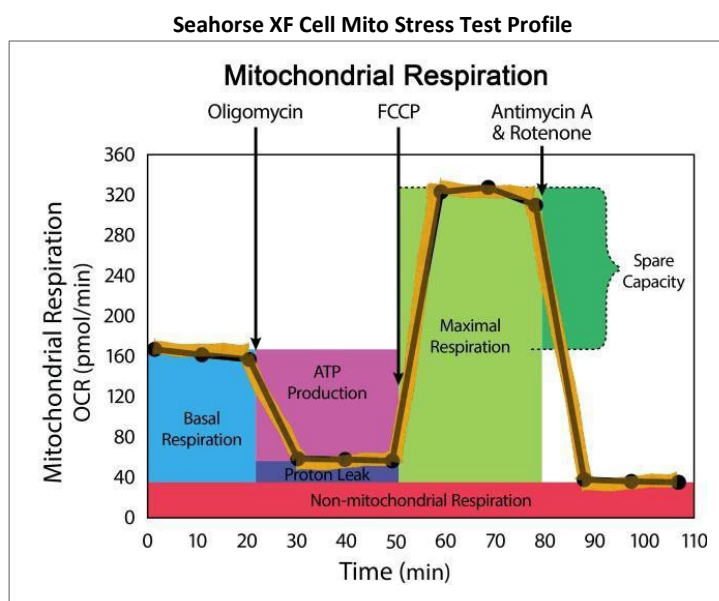
## Introduction

The Agilent Seahorse XF Stress Test Report Generators are the recommended tool for analysis of assay data and automatically calculate and report the key parameters of the XF Cell Mito and Glycolysis Stress Test assays.

Parameter values are calculated as absolute oxygen consumption rate (OCR) in pmol O<sub>2</sub>/min, or absolute extracellular acidification rate (ECAR) in mpH/min. The XF Stress Test Report Generators supports assay results from all Agilent Seahorse XF Analyzers.

## Parameter Calculations

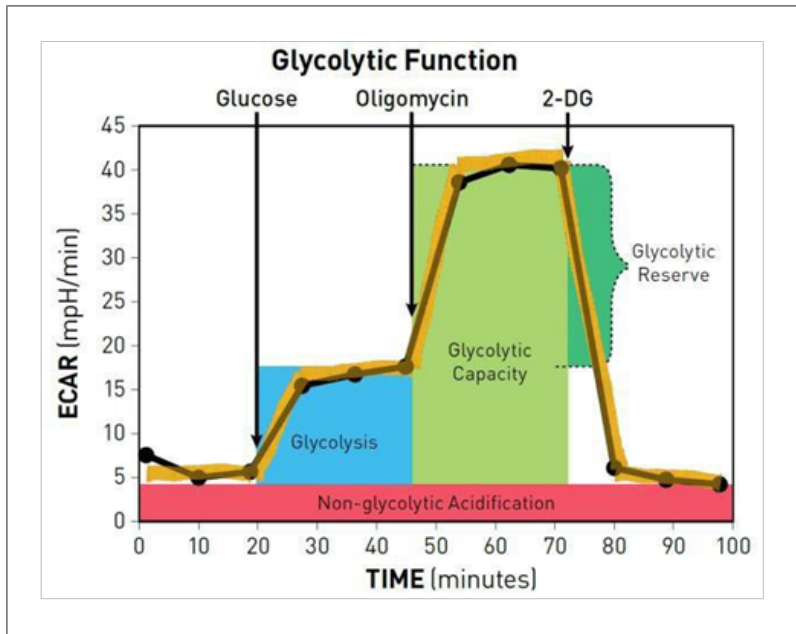
Parameter calculations performed in the XF Cell Mito and Glycolysis Stress Test Report Generators are outlined in Table 1 and Table 2 below. Each parameter value calculated represents the average of individual well calculations for each assay group on the Plate Map. Error bars are calculated based on the individual well calculations for each parameter. See the *Calculations* tab in the Report Generator for more information and example calculations.



**Table 1** | XF Cell Mito Stress Test Parameter Equations

Parameter Value	Equation
Non-mitochondrial Respiration	Minimum rate measurement after Rotenone/antimycin A injection
Basal Respiration	(Last rate measurement before first injection) – (Non-Mitochondrial Respiration Rate)
Maximal Respiration	(Maximum rate measurement after FCCP injection) – (Non-Mitochondrial Respiration)
H <sup>+</sup> (Proton) Leak	(Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial Respiration)
ATP Production	(Last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection)
Spare Respiratory Capacity	(Maximal Respiration) – (Basal Respiration)
Spare Respiratory Capacity as a %	(Maximal Respiration) / (Basal Respiration) × 100
Acute Response	(Last rate measurement before oligomycin Injection) – (Last rate measurement before acute injection)
Coupling Efficiency	ATP Production Rate) / (Basal Respiration Rate) × 100

### Seahorse XF Glycolysis Stress Test Profile



**Table 2 | XF Glycolysis Stress Test Parameter Equations**

Parameter Value	Equation
Glycolysis	(Maximum rate measurement before Oligomycin injection) – (Last rate measurement before Glucose injection)
Glycolytic Capacity	(Maximum rate measurement after Oligomycin injection) – (Last rate measurement before Glucose injection)
Glycolytic Reserve	(Glycolytic Capacity) – (Glycolysis)
Glycolytic Reserve as a %	(Glycolytic Capacity Rate) / (Glycolysis) × 100
Non-glycolytic Acidification	Last rate measurement prior to glucose injection
Acute Response	(Last measurement rate before glucose injection – Last rate measurement before acute injection)

## Configure Microsoft Excel to Enable Macros

The XF Cell Mito and Glycolysis Stress Test Report Generators are Microsoft Excel Macro-Enabled Worksheet and is compatible with Microsoft® Excel™ versions 2010, 2013 and 2016. In order to use the Report Generators, Excel must be configured to allow **macros** to run:

*To enable macros once:*

1. Double-click the *Seahorse XF Cell Mito or Glycolysis Stress Test Report Generator.xlsm* file icon.
2. Click **Enable Editing** and **Enable Content** (located on the yellow information bar) if prompted to do so when opening the Report Generator.

*To always enable macros (recommended for the best experience using Report Generators):*

1. Open Microsoft Excel.
2. Click **File**, then click **Options**.
3. Click **Trust Center**, then click **Trust Center Settings**.
4. Click **Macro Settings**.
5. Select **Enable all macros**.

## Overview: XF Stress Test Report Generators

The XF Cell Mito and Glycolysis Stress Test Report Generators display assay result data as well as additional information about the assay on 4-5 tabs. For the optimal Report Generator data analysis experience, [update to Wave Desktop 2.3](#).

1. **Summary Printout:** One-page graphical summary of the imported XF Stress Test assay result data presented as a kinetic graph and bar charts for select XF Stress Test parameters.
2. **Bar Charts:** OCR bar charts of the calculated parameter values for the specific XF Stress Test for each group displayed.
3. **Normalize:** View or edit normalization values applied to result data. See the [Wave Desktop User Guide](#) for more info.  
*Note: This tab is displayed for assay results that have been normalized in Wave Desktop 2.3 before exporting to the Report Generator. For assay result data that has not been normalized in Wave Desktop 2.3, then the **Normalize** tab will not be displayed.*
4. **Measures Sheet:** Table of parameter values for each group displayed in the Report Generator, ECAR kinetic graph, and coordinates for each group on the Plate Map.
5. **Calculations:** Overview of what rates are selected on the kinetic graph, equations, and how the well-to-well calculations are performed for each group analyzed.

## How To:

The following sections describe how to perform routine functions in the Report Generator:

- Analyze Data in the Report Generator:
  - Export from Wave Desktop 2.3 *\*Recommended\**
  - Import Excel file to Report Generator
  - Select Groups and Display Results
- Save a *Summary Report*.
- Normalize Assay Results:
  - Export from Wave Desktop 2.3 *\*Recommended\**
  - Import Excel file to Report Generator
- Exclude Outlier Wells:
  - Export from Wave Desktop 2.3 *\*Recommended\**
  - Import Excel file to Report Generator.

## Analyze Data in the Report Generator:

*\*Recommended\**

*After exporting from Wave Desktop 2.3:*

The recommended method to analyze assay result data in the Report Generator is using the **direct export** feature in Wave Desktop 2.3:

1. Transfer the assay result file to a personal computer using a USB flash drive or shared network directory.
2. Double-click to open the assay result file in Wave Desktop 2.3.
3. Click **Export**.
4. Select the **Seahorse XF Cell Mito Stress Test Report Generator** or the **XF Glycolysis Stress Test Report Generator**.  
*Optional: Modify the default file name and save location.*
5. Click **Save**.

*Import Excel file manually:*

To manually import assay result data to the Report Generator:

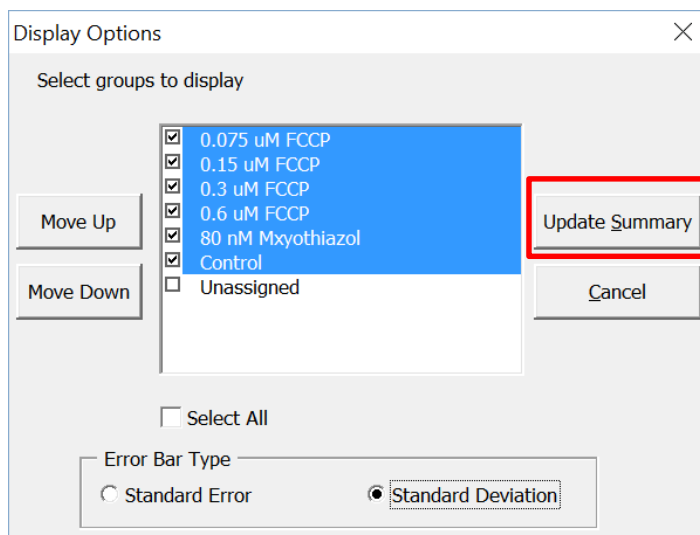
1. Click **Export**.
2. Select **Microsoft Excel**.  
*Optional: Modify the default file name and save location.*
3. Click **Save**.
4. Download the XF Stress Test Report Generators from the [Agilent website](#).
5. Unzip the compressed folder and open the Report Generator file.
6. Click **Load New Data File**.
7. Locate the Microsoft Excel file (exported from Wave Desktop) and click **Open**.

## Select Groups and Display Results

After exporting assay result data from Wave Desktop 2.3 to the Report Generator (or manually importing Excel data) into the Report Generator, use the *Display Options* dialogue window to select groups from the assay to display and click **Update Summary** (Figure 1). The Report Generator will automatically calculate the parameters for each group selected and display results on the *Summary Printout* tab.

### Error Bar Type

Standard Deviation is selected as the default *Error Bar Type* for ALL graphs. The *Error Bar Type* applies to all graphs in the Report Generator.



**Figure 1** | Group names configured in Wave will be displayed on the *Display Options* window when selecting groups in the Report Generator.

## Save a Summary Report:

*\*Recommended\**

*After exporting from Wave Desktop 2.3:*

Wave Desktop 2.3 automatically creates a unique *Summary Report* file (Microsoft Excel Macro-Enabled Worksheet) for each assay result file exported the Report Generator. Open the *Summary Report* file to view calculated parameters for selected groups, format/customize the appearance of graphs and figures, or select new groups from the assay result file to calculate assay parameters. Additional data files cannot be loaded into this Report Generator file as this file represents the Summary Report for *one* assay. Export additional assay result files from Wave Desktop 2.3 to the Report Generator to create multiple *Summary Reports*.

### *Import Excel file manually:*

Options for saving a Summary Report of assay result data that has been manually imported to the Report Generator include:

- **Save/Save as:** Click the *Save* icon (small floppy disc) to display the *Save as* function. Select a file location and enter a custom file name if desired. The saved Summary Report can be re-opened to view the calculated parameters for the selected groups, format/customize the appearance of graphs and figures or select new groups from the assay to run through the Report Generator. The Report Generator default file type is a *Microsoft Excel Macro-Enabled Template (\*.xltm)*. This file cannot be overwritten.
- **Save As – Excel Workbook:** Use the *Save As* function to save the customized Summary Report as an Excel Workbook file format (\*.xlsx).
- **Save As – PDF:** Use the *Save As* function to save the customized Summary Report as a PDF file format (\*.pdf).

*Note: Saving the Report Generator as an Excel workbook or any other file type than the default file type (Excel Macro: \*.xltm) will render the Report Generator macro inoperable – modifying the groups selected or importing additional assay result data is not supported in the Excel Workbook file format.*

## Advanced Options

Advanced Options is accessed on the *Display Options* window when selecting Assay Groups to display and displays the Instrument Protocol, which is automatically imported into the Report Generators from the Excel file. Instrument Protocol displays the number of:

- Injections performed in the imported Excel file from the Stress Test.
- Measurements before injection (Baseline) and after each injection from the Stress Test.

## Acute Injections

An injection that occurs in the Stress Test assay following the baseline measurements, but before the Oligomycin injection (*Seahorse XF Cell Mito Stress Test*) or Glucose injection (*Seahorse XF Glycolysis Stress Test*) is called an *Acute Injection*. This injection is automatically configured in the Seahorse Default Assay Templates for the Seahorse XF Cell Mito Stress Test (Acute Injection) and Seahorse XF Glycolysis Stress Test (Acute Injection). For custom assays, this injection step must be added manually in the Instrument Protocol in Wave (Desktop or Controller) prior to performing the Stress Test.

*Note: An Instrument Protocol with Custom Cycle steps are not supported in the Seahorse XF Stress Test Report Generators. See FAQs.*

- An acute injection must be performed **before** injection of the Stress Test compounds using Port A on the Cartridge.
- The Report Generators enable the **Acute Injection** checkbox by default when a 4<sup>th</sup> injection is detected in the imported Excel result file.
- For Stress Test assays with an **Acute Injection**, parameter calculations in Table 1 & Table 2 are shifted by the number of acute response measurements.
- Spare Respiratory Capacity (Seahorse XF Cell Mito Stress Test *only*) in the presence of an acute injection is calculated using the equation: (Maximal Respiration) – (Last Rate Measurement before Oligo Injection)

## Error Bars and Calculations

Error Bar Type is a universal setting and applies to ALL graphs and charts in the Report Generator. Standard Deviation is the default error bar type. To change the error bar type to Standard Error, click **Edit Current Group Selection** and select Standard Error.

- Error Bars are calculated from each replicate of the rate measurement used to determine the XF Stress Test parameter (see [Table 1](#)).
- Standard Deviation is calculated using the Microsoft Excel function.
- Standard Error of the Mean is calculated using the equation:  $\frac{(Standard\ Deviation\ of\ Group)}{\sqrt{(Number\ of\ Wells\ in\ Group)}}$

## Normalize Assay Results:

*\*Recommended\**

*After exporting from Wave Desktop 2.3:*

Normalize assay results to a cellular or mitochondrial parameter in the Report Generators. The simplest way to analyze normalized assay result data in the Report Generator is to first normalize the assay result data in Wave Desktop 2.3 (Figure 2a). Rate data that has been normalized in Wave Desktop 2.3 will be exported to the Report Generator and used for parameter calculations. View the **Normalize** tab to see raw normalization values, unit, and scale factor as entered in Wave Desktop 2.3 (Figure 2b). After selecting groups, normalized data is the default data displayed. Use the **Normalize** button on the *Summary Printout* page to toggle the data displayed between normalized and non-normalized rate data (Figure 2c).

Normalization Unit: Cell Count	
	1
A	
B	20000
C	20000
D	20000
E	20000
F	20000
G	20000
H	

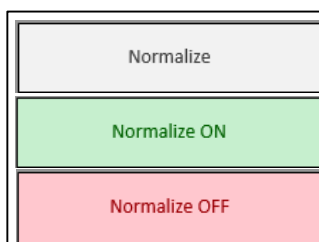
**Figure 2a** | Example of the Normalization Plate Map in Wave Desktop 2.3 for the Agilent Seahorse XFp Analyzer.

1	
A	0.00
B	20000.00
C	20000.00
D	20000.00
E	20000.00
F	20000.00
G	20000.00
H	0.00

Normalization Unit: Cell Count

Scale Factor: 1

**Figure 2b** | Example of the Normalization Plate Map in Wave Desktop 2.3 for the Agilent Seahorse XFp Analyzer.



**Figure 2c** | **Normalize** button on the *Summary Printout* tab. Data exported from Wave Desktop 2.3 without normalization values applied will show a gray **Normalize** button. By default, normalized rate data exported to the Report Generator from Wave Desktop 2.3 will be displayed, as indicated by the *Normalize ON* button status. Display non-normalized rate data by clicking the *Normalize OFF* button to display rate data without applying normalization values.

*Note: To preserve data integrity between Wave Desktop 2.3 and Report Generators, normalized data exported to a Report Generator is locked for editing. To modify the normalization values used in the Report Generator, they first must be edited in Wave and then re-exported to the Report Generator. Data exported to the Report Generator that is not normalized will not display the **Normalize** tab.*

### Import Excel file manually:

For versions of Wave 2.2 and earlier, assay result data must first be saved as an Excel Workbook file and manually imported to the Report Generator, then normalization values may be added to the **Normalize** tab in the Report Generator (Figure 3).

To add normalization values to the Report Generator:

1. Copy the normalization values from Wave or an external source such as another Excel file.
2. Open the Report Generator.
3. Click the **Normalize** tab.
4. Paste normalization values into the *Plate Map*.
5. Type in a *Normalization Unit*.
6. Click **Apply**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.00	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.00
B	4.41	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.91
C	4.41	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.91
D	4.41	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.91
E	4.41	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.91
F	4.41	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.91
G	4.41	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.91
H	1.00	4.41	4.41	4.40	4.40	3.81	3.81	3.20	3.20	1.91	1.91	1.00

Normalization Unit:

Scale Factor:

**Figure 3** | Example of the Normalization Plate Map after importing data from an Agilent Seahorse XFe96 or XF96 Analyzer.

### Optional: Copy Normalization Values from Wave Desktop:

If the kinetic data has been normalized in Wave Desktop or Wave Controller, Copy/Paste the normalization values into the Report Generator

In Wave (Desktop or Controller):

1. Open the Assay Result file (\*.asyr)
2. Click **Modify** (on any Analysis View), then click **Normalization**.
3. Press **Select All** to highlight the Plate Map in Wave.
4. Press **Ctrl + C** to copy the normalization values from the Wave > Normalization View.

*Note: Wave (Desktop and Controller) exports raw data only – Normalized rate data is not exported in the MS Excel output file.*

In the Report Generator:

1. Click the **Normalization** tab.
2. Click and highlight all wells in the Normalization Plate Map.
3. Press **Ctrl + V** to paste the copied normalization values from Wave into the Report Generator.
4. Repeat steps 2 and 3 for each Plate Map in the Report Generator.
5. Type in a **Normalization Unit**.
6. Press **Normalize Data**.
7. Click OK in the *Success* message box.

*Note: All wells on the Plate Map (Background and Experimental) must have a value of '1' entered into the Normalization Table. Values of '0' are not supported and will present an error message.*

## Exclude Outliers:

*\*Recommended\**



### After exporting from Wave Desktop 2.3:

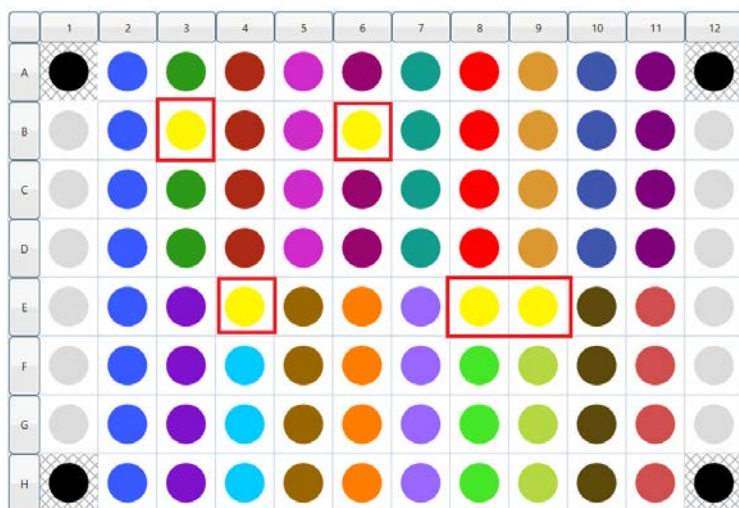
Assay wells and groups that are turned off in Wave Desktop will not be exported or included in parameter calculations for each group in the Report Generator. To turn off assay wells or groups prior to export, simply click the well or double-click the group on the **Group List** and that well or group will not be exported. The *Data* tab displays the group names, plate map layout, and any assay wells or groups that have been excluded from the export and group calculations in the Report Generator.

### Import Excel file manually:

Outliers or unwanted wells must be excluded from parameter calculations in the Report Generator **before** exporting assay result data to MS Excel from Wave (Desktop or Controller).

### Within Wave (Desktop or Controller):

1. Create 'Outlier' Group:
  - a. Open the Assay Result file in Wave (Desktop or Controller).
  - b. Click **Modify** (upper-right corner).
  - c. Click **Groups/Conditions**.
  - d. On the **Groups/Conditions** page, press **Add**  to add a new Group. Name the new group 'Outliers'.
  - e. Click **Apply**.
2. Assign Outlier Wells to 'Outlier' Group:
  - a. Click **Modify** (upper-right corner).
  - b. Click **Plate Map**.
  - c. Locate the group name 'Outlier' from the list of groups on the left, then click the group name to select the 'Outlier' group. 
  - d. Click the wells on the Plate Map to assign to them to the 'Outlier' group. Below is an example of Outlier wells assigned to the Plate Map outlined in red (Figure 4).
  - e. Click **Apply**.
  - f. Click **Save As** to save the Assay Result data as a MS Excel Workbook (\*.xlsx). See section called *Step II – Save Wave Assay Result File in Excel format* (Page 3) for steps on saving data as an Excel Workbook (\*.xlsx).



**Figure 4** | Plate Map view in Wave. The wells with a red outline have been reassigned to the 'Outlier' group (denoted by the yellow group color).

#### HELPFUL HINTS:

Unassign Assay Wells in Wave: A quicker method to remove outliers for Report Generator analysis is to unassign assay wells. To do this, open the Assay Result file in Wave and click **Modify**. Next, click **Plate Map** and simply click each assay well that you want to *unassign*. Unassigned assay wells will turn gray. Click **Apply** and Wave will automatically create an *Unassigned* group. Proceed to *Step f.* on the next page.

Record Coordinates: After reviewing data but before modifying **Groups/Conditions** or **Plate Map**, write down the coordinates of the outliers on a piece of paper. Example: The yellow outlier well coordinates in Figure 4 are: B3, B6, E4, E8, and E9.

## Glossary

### **Oxygen Consumption Rate (OCR)**

The rate of decrease of oxygen concentration in the assay medium. OCR is a measure of the rate of mitochondrial respiration of the cells.

### **Extracellular Acidification Rate (ECAR)**

The rate of increase in proton concentration [or decrease in pH] in the assay medium. OCR is a measure of the rate of glycolysis of the cells.

### **Basal Respiration**

Oxygen consumption used to meet cellular ATP demand and resulting from mitochondrial proton leak. Shows energetic demand of the cell under baseline conditions.

### **ATP Production**

The decrease in oxygen consumption rate upon injection of the ATP synthase inhibitor oligomycin represents the portion of basal respiration that was being used to drive ATP production. Shows ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell

### **H<sup>+</sup> (Proton) Leak**

Remaining basal respiration not coupled to ATP production. Proton leak can be a sign of mitochondrial damage or can be used as a mechanism to regulate the mitochondrial ATP production.

### **Maximal Respiration**

The maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological “energy demand” by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, amino acids) to meet this metabolic challenge. Shows the maximum rate of respiration that the cell can achieve.

### **Spare Respiratory Capacity**

This measurement indicates the capability of the cell to respond to an energetic demand as well as how closely the cell is to respiring to its theoretical maximum. The cell’s ability to respond to demand can be an indicator of cell fitness or flexibility.

### **Non-mitochondrial Respiration**

Oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after rotenone and antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration.

## Frequently Asked Questions

### **How do I remove outlier wells in the Report Generator?**

Outliers must be removed prior to data analysis in the Report Generator. See section called [Exclude Outliers](#) for more information.

### **I’m unable to import my data as an Excel file into the Report Generator for analysis, why?**

Assay result data must be exported from Wave (Desktop or Controller) or from XFp 1.1 software as an Excel Workbook file format (\*.xlsx) before importing into any Report Generator. If the Excel file has been exported from Wave but cannot be imported to the Report Generator, please contact Seahorse Technical Support at: [seahorse.support@agilent.com](mailto:seahorse.support@agilent.com)

### **If you receive an error message about Instrument Protocol (XF<sup>e</sup>96; XF<sup>e</sup>24; XF96; XF24 only)**

Errors upon data import into the Report Generator are likely caused by a *custom cycle* in your Instrument Protocol. A *Custom Cycle* refers to an additional 'Mix' or 'Wait' command step in the Instrument Protocol an assay. Custom Cycles are not part of the standardized assay template for the XF Cell Mito and Glycolysis Stress Tests and are not supported in Report Generator analysis. Please contact Seahorse Technical Support if you have any additional questions regarding Custom Cycles.

### **Where is my ECAR data?** (XF Cell Mito Stress Test Report Generator *only*)

ECAR (Extracellular Acidification Rate) data is displayed on the *Measures Sheet* tab in the XF Cell Mito Stress Test Report Generator.

### **Where is my OCR data?** (XF Glycolysis Stress Test Report Generator *only*)

OCR (Oxygen Consumption Rate) data is displayed on the *Measures Sheet* tab in the XF Glycolysis Stress Test Report Generator.

### **How do I combine multiple result files in this Report Generator?**

The XF Stress Test Report Generators enable analysis of individual assay result files. Combining multiple result files in the Report Generators is not supported at this time.

### **What rate measurements are used to calculate the parameters in this Report Generator?**

Parameter equations are described in [Table 1](#) of this User Guide.

## **Feedback**

Feedback for the Report Generator or other products is always encouraged. Please direct any questions, concerns or suggestions to Seahorse Technical Support at: [seahorse.support@agilent.com](mailto:seahorse.support@agilent.com)

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