Report Generator
User Guide

Agilent Seahorse
XF Cell Mito Stress Test

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Introduction

Agilent Seahorse XF data analysis and interpretation – **accelerated**. In a few simple steps, transform Agilent Seahorse XF Cell Mito Stress Test assay result data into interpretable, compelling data tables and figures of mitochondrial function for each group analyzed using the Agilent Seahorse XF Cell Mito Stress Test Report Generator. Easily copy figures or data tables from the Report Generator to other software programs for additional graphing or statistical analysis and eliminate tedious, error-prone steps in any XF data analysis workflow.

The Seahorse XF Cell Mito Stress Test Report Generator is a Microsoft Excel Macro that automatically calculates and reports assay parameters of the Seahorse XF Cell Mito Stress Test (Table 1) as absolute Oxygen Consumption Rate (OCR) in pmol O_2/min and supports assay result data from all Agilent Seahorse XF Analyzers.

Parameter Calculations

Parameter calculations performed in the Seahorse XF Cell Mito Stress Test Report Generator are outlined in Table 1 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.
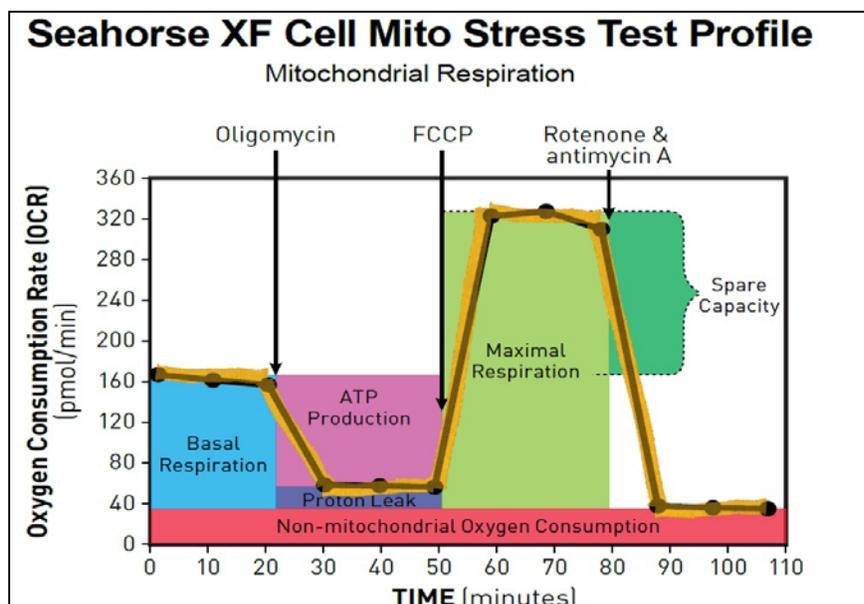

Figure 1 | Agilent Seahorse XF Cell Mito Stress Test assay parameters and kinetic profile.

Table 1 | Seahorse XF Cell Mito Stress Test Parameter Equations

Parameter Value	Equation
Non-mitochondrial Oxygen Consumption	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+ (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

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XF Report Generator Overview

The Seahorse XF Cell Mito Stress Test Report Generator displays result data and other assay-related information on 5-6 tabs.

For the optimal Report Generator data analysis experience, update to Wave 2.3.

1. **Summary Printout:** One-page *Summary Report* of the imported Seahorse XF Cell Mito Stress Test result data presented as a kinetic graph and bar charts for select assay parameters.
2. **Bar Charts**: Assay parameter data displayed as bar charts.
3. **Normalize**: Plate map of normalization values applied in the imported result file. This tab is only displayed for result files that have been normalized in Wave 2.3 before Excel export. See the Wave User Guide for more info.
   *Note: Assay result data that has not been normalized in Wave 2.3 will not be display the **Normalize** tab.*

4. **Measures Sheet**: Result data organized in tabular format for assay parameters and kinetic rate data for both OCR and ECAR.
5. **Assay Parameter per Well**: Data table of assay parameters per well for each group in the imported file.
6. **Sample Calculations**: Example parameter calculations using sample data.

How To:

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

- Analyze Data in the Report Generator
- Save a *Summary Report*
- Normalize Assay Results
- Exclude Outlier Wells

Configure Microsoft Excel to Enable Macros

The Seahorse XF Cell Mito Stress Test Report Generator is a Microsoft Excel Macro-Enabled Template and is compatible with Microsoft® Excel™ versions 2010, 2013 and 2016 for Windows PCs, and Microsoft® Excel™ for Mac versions 2011 and 2015. In order to use this Report Generator, Excel must be configured to allow **macros** to run. To enable macros once, double-click the *Multi-File_Seahorse XF Cell Mito Stress Test Report Generator.xltm* file, then click **Enable Editing** and **Enable Content** (yellow information bar) if prompted (Figure 2).

Figure 2 | Enable macros using the **Enable Editing** button as seen on the yellow information bar. This needs to be performed once upon opening the Report Generator for the first time after download.

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**.

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Analyze Data in the Report Generator:

Recommended
Direct-export from Wave 2.3:

The recommended method to analyze assay result data in the Report Generator is using the **direct export** from Wave 2.3 (Figure 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**.
   Optional: Modify the default file name and save location.
5. Click **Save**.

Import Excel file manually:

1. In Wave 2.3, click **Export**.
2. Select **Microsoft Excel** and click **Save**.
   Optional: Modify the default file name and save location.
3. Download the Seahorse XF Cell Mito Stress Test Report Generators from the Agilent website.
4. Unzip the compressed folder and open the Report Generator file.
5. Click **Load New Data File**.
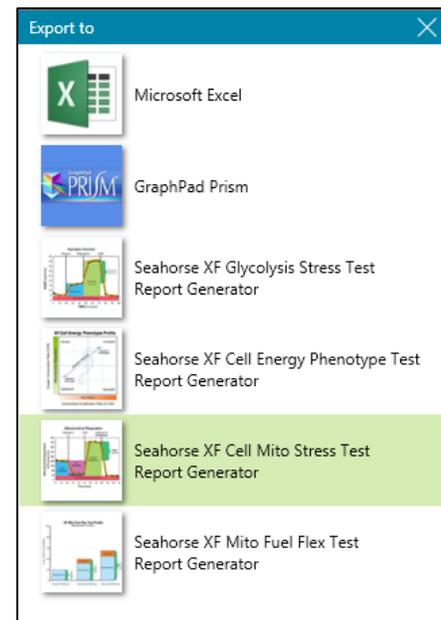6. Locate the Microsoft Excel file (exported from Wave 2.3) and click **Open**.

Figure 3 | Wave 2.3 (Desktop & Controller) export options.

Select Groups and Display Results

After exporting assay result data from Wave 2.3 to the Report Generator (or manually importing Excel data), use the *Display Options* dialogue window to select groups from the assay to display and click **Update Summary** (Figure 4). 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.
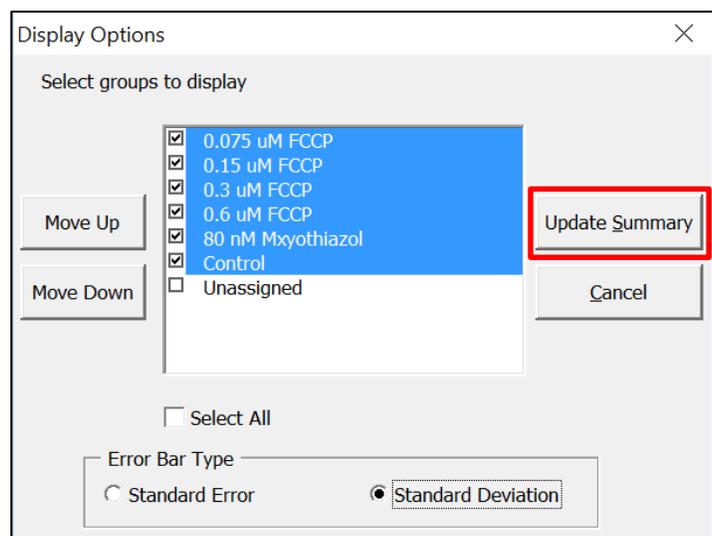

Figure 4 | Group names configured in Wave shown on the *Display Options* window when selecting groups in the Report Generator.

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Save a Summary Report:
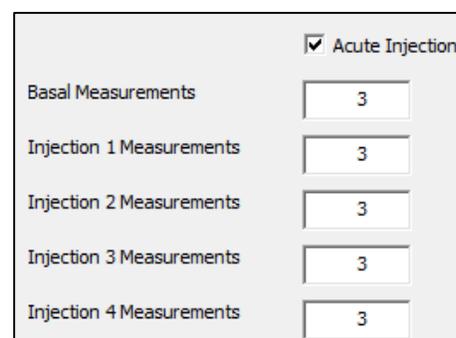
Recommended
After exporting from Wave 2.3:

When exporting data from Wave 2.3 to the Report Generator, Wave will automatically create a unique *Summary Report* file (Microsoft Excel Macro-Enabled Worksheet) for each exported result file. Additional data files cannot be loaded into this Report Generator file and must be created using the direct-export from Wave 2.3.

- **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: *.xlsm) will render the Report Generator macro inoperable – modifying the groups selected or importing additional assay result data is not supported as an Excel Workbook file type.

Advanced Options

Advanced Options (Figure 5) is accessed from the *Display Options* window when selecting groups to display by clicking **Advanced**. *Advanced Options* displays the Instrument Protocol, which is automatically imported into the Report Generator from Wave or the Excel file. Instrument Protocol displays the number of injections performed file and measurements before injection and after each injection.

Figure 5 | Instrument Protocol displayed in *Advanced Options.*

Acute Injections

An injection that occurs in the Seahorse XF Cell Mito Stress Test assay following the baseline measurements, but before the Oligomycin injection is called an *Acute Injection*. The default Seahorse assay template in Wave (Desktop or Controller) called "Seahorse XF Cell Mito Stress Test (Acute Injection)" has the specific Instrument Protocol for acute injections and should be used for every Seahorse XF Cell Mito Stress Test assay with an acute injection. For custom assays, this injection step must be manually added to the Instrument Protocol in Wave (Desktop or Controller) prior to the assay.

- An acute injection must be injected **before** oligomycin from Port A on the cartridge.
- The **Acute Injection** checkbox is enabled automatically when a 4th injection is detected in the imported Excel file (Figure 5).
- Parameter calculations in Table 1 are shifted by the number of acute response measurements for assays with an **Acute Injection**.
- Spare Respiratory Capacity in the presence of an acute injection is calculated using the equation:
  (Maximal Respiration) − (Last Rate Measurement before Oligo Injection)

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Error Bar 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 of the Mean (S.E.M.), click **Edit Current Group Selection** and select Standard Error.

- Error Bars are calculated from each replicate of the rate measurement used to determine the assay parameter (see Table 1).
- Standard Deviation is calculated using the Microsoft Excel function.
- S.E.M. is calculated using the equation: $\frac{(Standard\ Deviation\ of\ Group)}{\sqrt{(Number\ of\ Wells\ in\ Group)}}$

Normalize Assay Results:

It is highly recommended to analyze rate data that has been normalized to a cellular or mitochondrial parameter compared to non-normalized, raw rate data. Normalization data must be added to the **Normalize** view in Wave 2.3 (Figure 6a) before export — normalized rate data will be used for parameter calculations and displayed by default in all kinetic graphs and bar charts in the Report Generator. Click the **Normalize** tab to view the normalization plate map, unit, and scale factor as entered in Wave 2.3 (Figure 6b). Use the **Normalize** button on the *Summary Printout* page to toggle the data displayed on each chart between normalized and non-normalized rate data (Figure 6c).

Figure 6a | Example of a normalization plate map for the Agilent Seahorse XFp Analyzer in Wave 2.3.

Figure 6b | The same normalization plate map from Wave 2.3 (Figure 6a) displayed in the Report Generator.
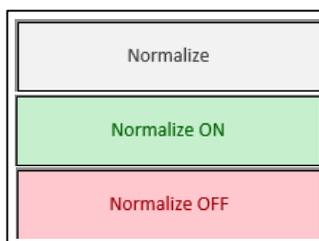
Figure 6c | Normalize button on the *Summary Printout* tab. By default, normalized rate data exported from Wave will be displayed as indicated by the *Normalize ON* button status (green). Click the *Normalize ON* button to toggle the data display to show non-normalized rate data (*Normalize OFF* – red). Data exported from Wave 2.3 without normalized rate data will show a gray **Normalize** button.

*Note: To preserve data integrity between Wave 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 2.3 and then re-exported to the Report Generator. If result data has not been normalized, the Report Generator will not display the **Normalize** tab.*

Exclude Assay Wells (Outliers) or Groups from Report Generator Analysis:

Individual assay wells or entire groups/conditions can be excluded from parameter calculations in the Report Generator. Before exporting data from Wave 2.3, click the assay well(s) on the plate map or double-click the group name(s) on the **Group List** to exclude those wells or groups from data export. The *Project Information* tab displays the plate map layout and any assay wells or groups that have been excluded from the Excel export (Figure 7).

Figure 7 | XFp Plate Map on the *Project Information* tab. Assay well C has been turned off in Wave 2.3 prior to export, therefore the *Control* group parameter calculations are based on assay wells B and D only.

Frequently Asked Questions

What rate measurements are used to calculate the parameters in this Report Generator?
Parameter equations are described in Table 1 of this User Guide.

How do I remove outlier wells in the Report Generator?
Outlier assay wells must be turned OFF or reassigned in Wave 2.3 prior to Excel export. See section called Exclude Outliers/Groups from Analysis or the Wave User Guide for more information.

Can I use the Excel file exported from the XFp, XF96, or XF24 Analyzer?
It is recommended to export assay result data directly to the XF Report Generator from Wave 2.3 (Desktop or Controller), however data can also be manually imported as an Excel file (*.xlsx). Excel files exported from earlier versions of Wave (Desktop or Controller) and XF96/XF24 software are not compatible. If the Excel file has been exported from Wave but cannot be imported to the Report Generator, please contact Seahorse Technical Support.

If you receive an error message about Instrument Protocol (XFe96; XFe24; XF96; XF24 only)
Errors upon data import into the Report Generator are likely caused by a c*ustom cycle* in the 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 Seahorse XF Cell Mito Stress Test and not supported in Report Generator analysis. Please contact Seahorse Technical Support if you have any additional questions regarding Custom Cycles.

Can I use baseline rate data (%) to calculate assay parameters?
Normalized (or non-normalized) OCR data is used for parameter calculations, parameter calculations using baseline rate data (%) is not supported at this time.

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

How do I analyze multiple result files in a Report Generator?
The Seahorse Multi-File XF Cell Mito Stress Test Report Generator enables the import and analysis of multiple XF Cell Mito Stress Test assay result files. Find out more and download the Multi-File XF Report Generator **here**.

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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

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+ (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 Oxygen Consumption

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.

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