# Multi-File XF Report Generator

# User Guide

Agilent Seahorse

XF Cell Mito Stress Test

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# Introduction

Seahorse XF data analysis and interpretation – **accelerated**. In a few simple steps, transform 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 Seahorse Multi-File XF Cell Mito Stress Test Report Generator. Easily copy figures or data tables from the Multi-File XF 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 Multi-File XF Report Generator is a Microsoft Excel Macro that automatically calculates and reports assay parameters of the Seahorse XF Cell Mito Stress Test (Table 1) absolute Oxygen Consumption Rate (OCR) in pmol $O_2$/min and supports assay result data from all Agilent Seahorse XF Analyzers.

# Parameter Equations

Assay parameters of the Seahorse XF Cell Mito Stress Test assay along with parameter equations are displayed in Table 1 below. The Multi-File XF Report Generator automatically reports assay parameters for each *Group Collection* configured from the imported Excel (.xlsx) files.
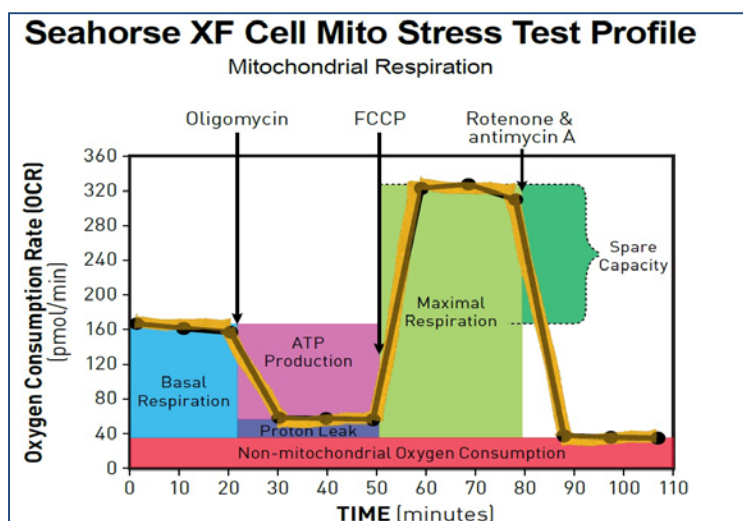


**Table 1 |** XF Cell Mito Stress Test Parameter Equations

| 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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# Parameter Calculations

The Multi-File XF Report Generator automatically calculates assay parameters of the Seahorse XF Cell Mito Stress Test (Table 1) as the average ± S.E.M of the average parameter value of the replicate groups assigned to a *Group Collection*. A *Group Collection* is defined as the collection of replicate groups (same treatment or condition) measured in independently repeated experiments over multiple plates. The 3 types of data formats are:

- Kinetic Graphs – OCR versus Time (*Summary Printout*); ECAR versus Time (*Measures Sheet*).
- Assay Parameter Bar Charts – OCR for each *Group Collection*.
- Data Tables – OCR for (a) individual rate measurement data on kinetic graph, (b) average assay parameter data for each group assigned to a *Group Collection*, (c) assay parameters per well for each group assigned to a *Group Collection*.

The Report Generator does not perform any statistical analysis (ANOVA; t-test, etc.) using imported data, for more information on this type of analysis please see the GraphPad Statistics Guide (H.J. Motulsky).

**Example Calculation using Group Collection 1: '*Group A*':**
In Figure 1 (below) the two treatment groups, Group A and Group B, are measured in 3 independent, repeated experiments – Plate 1, Plate 2, Plate 3.

**Figure 1|** Two treatment groups – Group A and Group B – measured in 3 independent experiments.
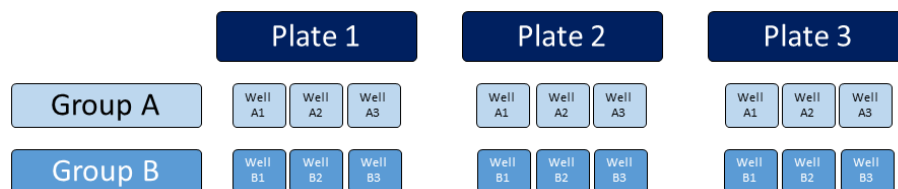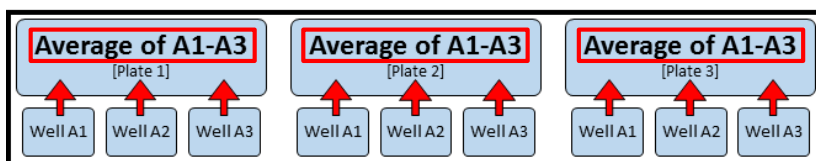


First, the Multi-File XF Report Generator calculates assay parameters within each well of the replicate groups assigned to the *Group Collection* (Figure 2).
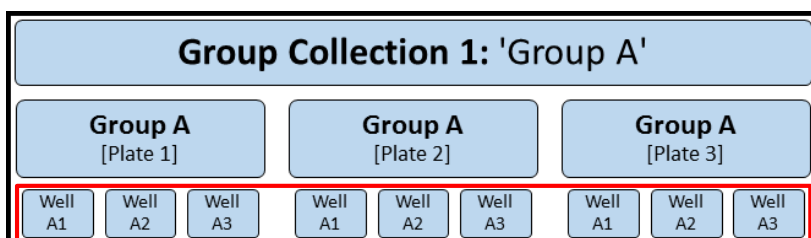


**Figure 2|** Assay parameters are calculated for each well (A1, A2, A3) in the groups assigned to the "Group A" *Group Collection*.

Next, the average parameter value for each replicate plate in the *Group Collection* is calculated based on the individual well parameter values (Figure 3).



**Figure 3|** The average assay parameter is calculated using the individual well parameter values for each group in the "Group A *Group Collection*.

Finally, the Multi-File XF Report Generator calculates the average ± S.E.M. based on the average parameter values of the groups in the *Group Collection* (Figure 4).
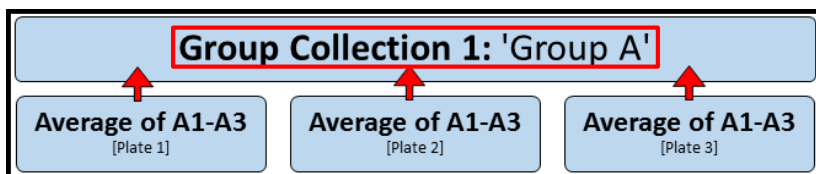


**Figure 4|** The final reported assay parameters represent the average ± S.E.M. of the average parameter value from each replicate group assigned to the Group A *Group Collection*.

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## Multi-File XF Report Generator – Technical Requirements & Best Practices

- Each assay result file must have **identical** *Instrument Protocols* (Mix, Wait, and Measure times).
- Each assay result file must be produced on the same Agilent Seahorse XF, XFe or XFp Analyzer.
- Two replicate groups must be assigned to create a *Group Collection*.
- Two groups from the same Excel file cannot be assigned to a *Group Collection*.
- Create a 'master' assay template in Wave 2.3 (Desktop or Controller) that will be used to perform each replicate Seahorse XF Cell Mito Stress Test assay. Using the same assay template for each experiment ensures each Excel file imported for analysis maintains consistent group naming, instrument protocol, and other important assay-related information.
- Apply normalization data in Wave 2.3 (Desktop or Controller) before exporting to Excel to report normalized rate data for analysis in the Multi-File XF Report Generator.
- It is recommended to assign at least 3 wells for each treatment or condition group in the assay template file.
- It is recommended to measure the same experimental groups/conditions in each replicate experiment.
- Be sure to inspect and analyze the kinetic graph – review the kinetic graphs for each independent assay result file in Wave software first to look for significant differences between replicate groups before calculating assay parameters in the Multi-File XF Report Generator.

## Multi-File XF Report Generator – Biological Best Practices

- Cell seeding density should be optimal and within the linear range of the cell activity as measured by OCR and/or ECAR. Optimal density may be obtained either via empirical assay or recommended values found in the XF Cell Reference Database, XF Publications Database, or XF Assay Template/Guides.
- Maintain consistent cell culture and/or subculture of cells, including but not limited to passage number, sub-culture density, serum lot, etc for each replicate experiment.
- Maintain consistent time of cell growth after seeding and consistent time of treatment or intervention for each replicate experiment.
- The concentration of FCCP (or any acutely injected compounds) must be optimized. Optimal concentrations of FCCP can be obtained via empirical assay or using the recommended values found in the XF Cell Reference Database, XF Publications Database, or XF Assay Template/Guides. The concentration of oligomycin and rotenone/antimycin-A in the Seahorse XF Cell Mito Stress Test Kit are pre-optimized.
- Ensure the cells maintain a consistent baseline rate in absence of any treatments and/or injected compounds. The baseline rate is defined as the rate measurements before compound injection from Port A (oligomycin or acute injection).
- Well-to-well consistency within experimental groups should be reviewed in Wave 2.3 for consistent cell seeding technique. Use the **Display** toggle (above the kinetic graph) to display kinetic rate data in **Well** mode to assist in diagnosing well-to-well variability within a single group.

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## Multi-File XF Report Generator Overview

Wave 2.3 (Desktop and Controller) is required to use the Multi-File XF Report Generator. Earlier versions of Wave software are not compatible. The Multi-File XF Cell Mito Stress Test Report Generator displays assay result data and other assay-related information on 6 tabs:

1. **Summary Printout:** One-page Summary Report of the selected *Group Collections* presented as a kinetic graph and bar charts for select assay parameters.
2. **Bar Charts**: Assay parameters displayed as bar charts for each *Group Collection*.
3. **Normalize**: Plate maps of normalization values for each imported Excel file. This tab is only displayed for result files that have been normalized in Wave 2.3 before exporting to Excel. See the Wave User Guide for more info.
4. **Measures Sheet**: Data table of average assay parameter values per file, data table of average OCR rate data and corresponding SEM, and ECAR kinetic graph, for each *Group Collection*
5. **Assay Parameter per Well**: Data table of assay parameters per well for each *Group Collection*.
6. **Project Information**: Details of the imported files, Group Collections, Plate Maps, and Software License Terms.

## How To:

The following sections describe how to perform routine functions in the Report Generator:
- Analyze Data in the Multi-File XF Report Generator
- Save a *Summary Report*
- Normalize Assay Results
- Exclude Outlier/Groups from Analysis Wells

## Configure Microsoft Excel to Enable Macros

The Seahorse Multi-File 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, 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.

*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 Multi-File XF Report Generator:

The 5 simple steps to compile and analyze multiple data files in the Multi-File XF Report Generator are outlined below.

### Step 1 – Download the Seahorse Multi-File XF Cell Mito Stress Test Report Generator:

1. Using any personal computer with internet access, visit the Report Generator download page on the Agilent website and register to download the Seahorse XF Cell Mito Stress Test Report Generators.
2. Once the download is complete, unzip the compressed folder (right-click the folder and select **Extract all...**).
3. The download includes both the Single-File and Multi-File XF Cell Mito Stress Test Report Generators.

### Step 2 – Export assay result data as Excel Workbook files (.xlsx) using Wave 2.3:

1. Using Wave 2.3 (Desktop or Controller), open an assay result file and click **Export**.
2. Select **Microsoft Excel** and click **Save**.
3. Repeat for each assay result file that will be analyzed using the Multi-File XF Report Generator.

### Step 3 – Import Excel Workbook files (.xlsx) to the Multi-File XF Report Generator:

1. Double-click the *Multi-File_Seahorse XF Cell Mito Stress Test Report Generator.xltm* file icon.
2. Click **Enable Editing** and **Enable Content** (located on the yellow information bar) after opening the Report Generator (if prompted).
3. Click **Load New Data Files**.
4. Locate the Microsoft Excel files exported from Wave 2.3 (Desktop or Controller).
5. Hold down the **Ctrl** key then left-click to select each Excel file to import for analysis. Click **OK** when finished.
6. The **File Options** window will appear (Figure 5) displaying the selected Excel files for analysis. Use the **Add Another File** or **Delete** button to add Excel files to the list, or remove Excel files from the list. Click **Continue** when ready.
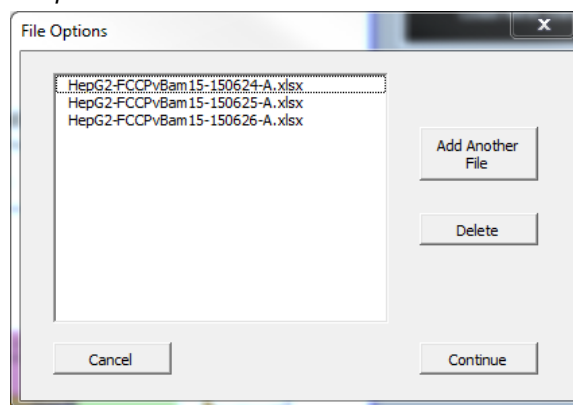


**Figure 5 |** *File Options* window displaying imported Excel files for analysis.

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## Step 4 – Configure *Group Collections*:

The *Configure Group Collections* window displays the list of groups/conditions for each imported assay result file as defined in the assay template used to generate the results (Figure 6).

*Note:* It is critical that the correct replicate group or condition from each file is added to the same Group Collection as the parameter calculations will be generated using the groups assigned to each Group Collection. See *Technical Requirements & Best Practices* for more information.

1. Create a *Group Collection* by assigning the replicate group/condition from each imported Excel file. Figure 6 below displays an example *Group Collection* comprised of 3 replicate groups – "B, 0.3" – measured in 3 independent assays.

2. When finished assigning groups to a *Group Collection*:
   a. Click **Continue** to proceed to generate a Summary Report.
   b. Click **Create another Group Collection** to configure additional groups/conditions to display in the Summary Report.
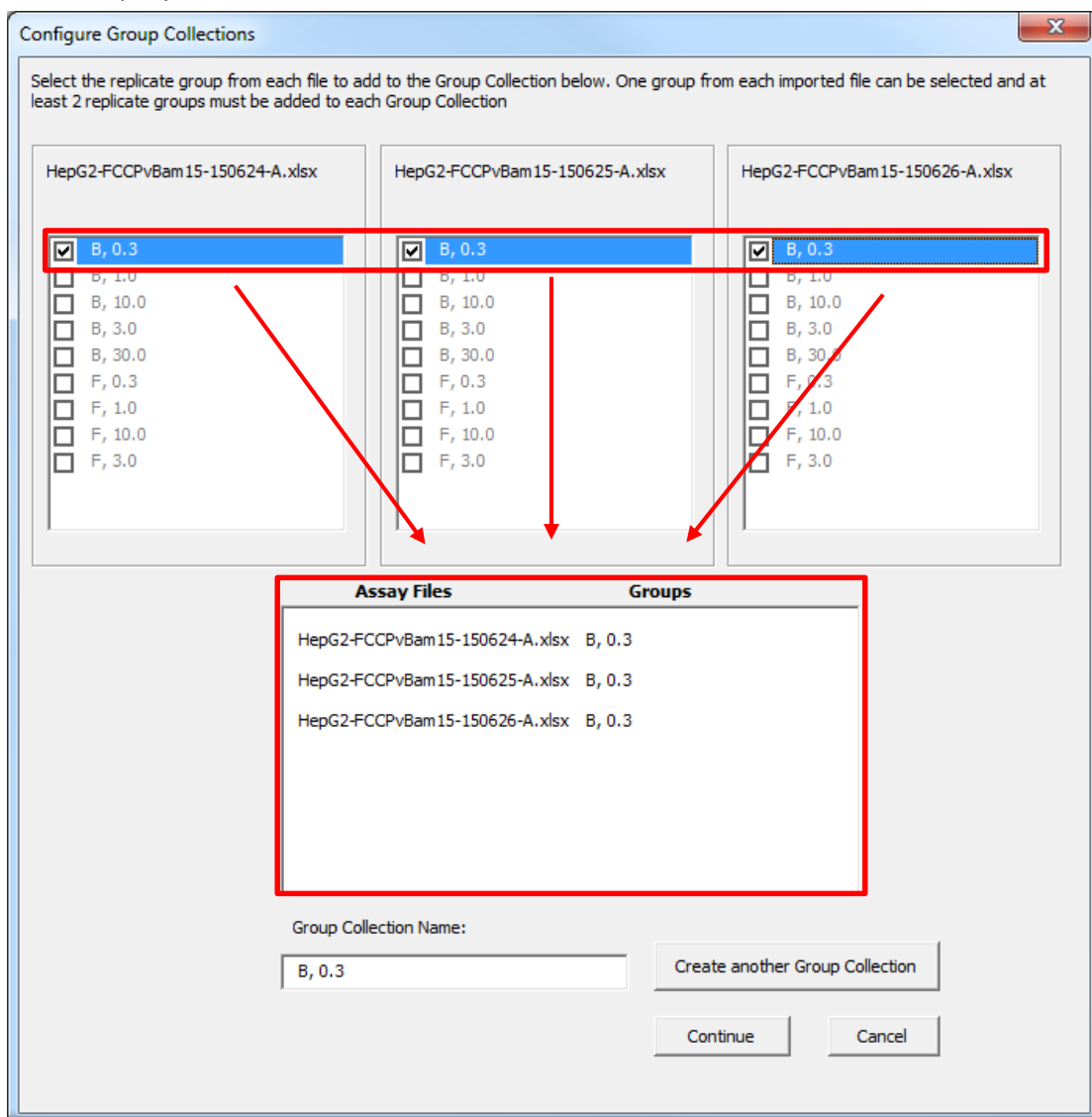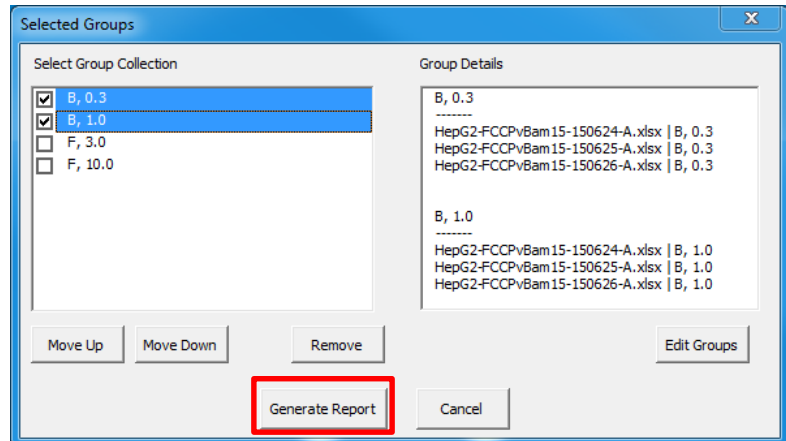


**Figure 6** | *Configure Group Collections* window. Check the box next to each replicate group or conditions within the imported Excel file. Select groups and the corresponding Excel file are displayed at the bottom of the window.

### Step 5 – Select Group Collections and Generate a Report:

Use the checkboxes to select the *Group Collections* that will be analyzed with the Multi-File XF Report Generator then click **Generate Report** (Figure 7).

The Group Details box displays the source file and individual group assigned to each selected *Group Collection*. If a group is mistakenly assigned to the wrong *Group Collection*, click **Edit Groups** to return to the *Configure Group Collections* window and modify the selected groups assigned to the Group Collection.



**Figure 7 |** List of configured *Group Collections* and details of each selected *Group Collection* within the Group Details box. Use the **Move Up/Move Down** buttons to modify the order of *Group Collections* when viewing results in the Multi-File XF Report Generator. Click **Generate Report** to create a *Summary Report* of the selected *Group Collections*.

## Save a Summary Report:

- **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 calculated parameters for each *Group Collection*, format/customize the appearance of graphs and figures, modify selected *Group Collections,* or upload additional Excel files for analysis. The default Report Generator file 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.*

## 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 any 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.

- Each imported Excel file must have identical Instrument Protocols – same number of injections, number of measurements, etc. – if a file is detected to have a mismatching Instrument Protocol compared to other imported files, it will not be imported.
- An acute injection must be injected **before** oligomycin from Port A on the cartridge.
- Parameter calculations (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)

## Error Bar Calculations

Standard Error of the Mean (S.E.M.) is the only error type in the Multi-File XF Report Generator and is calculated and displayed on every kinetic graph and bar chart for the configured *Group Collections*. S.E.M. is reported for each *Group Collection* and calculated using the equation:

$$\frac{(Standard\ Deviation)}{\sqrt{(n)}}$$

The *Standard Deviation* is calculated using the average group parameter values assigned to a *Group Collection* and *n* = number of groups assigned to a *Group Collection*, see Figure 8 for an example S.E.M. calculation.

**"Group A" Group Collection:**
- File #1/Group A --- Average Basal Respiration = 100 pmol/min
- File #2/Group A --- Average Basal Respiration = 110 pmol/min
- File #3/Group A --- Average Basal Respiration = 105 pmol/min

$$Basal\ Respiration\ S.E.M. = \frac{Standard\ Deviation\ [100, 110, 105]}{\sqrt{[3]}} = \frac{5}{1.73} = \ \pm\ 2.89$$

**Figure 8 |** Example Basal Respiration (OCR) S.E.M. calculation for "Group A" *Group Collection*.

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## 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 for each assay result file using Wave 2.3 (Figure 9a) before export, normalized rate data will be displayed by default for the kinetic rate data and assay parameter calculations in the Multi-File XF Report Generator. Click the **Normalize** tab to view the normalization plate map, unit, and scale factor as entered in Wave 2.3 (Figure 9b). Normalized rates are the default rate data displayed on each tab after selecting *Group Collections*. 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 9c).



**Figure 9a |** Example of a normalization plate map for the Agilent Seahorse XFp Analyzer in Wave 2.3.



**Figure 9b |** Example of a normalization plate map for the Agilent Seahorse XFp Analyzer in the Report Generator.
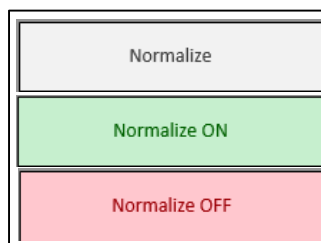


**Figure 9c | Normalize** button on the *Summary Printout* tab. Data exported from Wave 2.3 without normalized rate data will show a gray **Normalize** button. By default, normalized rate data will be displayed, as indicated by the *Normalize ON* button status, in the Report Generators. Click the *Normalize ON* button to toggle the data display to show non-normalized rate data.

*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 as Excel files. Excel files imported to the Multi-File XF Report Generator that are not normalized will not display the **Normalize** tab.*

## Exclude Outliers/Groups from Analysis:

Individual assay wells or entire groups/conditions can be excluded from parameter calculations in the Report Generator. Before exporting data to Excel from Wave, click the assay well(s) or double-click the group(s) on the **Group List** to turn off those wells/groups in Wave, then export data to Excel. The *Project Information* tab displays the plate map layout (Figure 10a) and any assay wells or groups that have been excluded from the Excel export (Figure 10b).



**Figure 10a |** XFp Plate Map on the *Project Information* tab. Assay well C has been turned off in Wave 2.3 prior to Excel export, therefore the *Control* group parameter calculations are based on assay wells B and D only.



**Figure 10b |** Formatting legend for the Plate Map(s) to indicate the assay wells that are assigned, unselected, and unassigned for parameter calculations.

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# Frequently Asked Questions

**What rate measurements are used to calculate assay parameters?**

Parameter equations are described in Table 1 of this User Guide.

**What rate measurements are used to calculate assay parameters?**

Parameter equations are described in Table 1 of this User Guide.

**How do I remove wells from the Report Generator calculations?**

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.

**How many Excel files can be imported for analysis?**

- XF/XFe96 & XF/XFe24 – A maximum of 5 Excel files (*.xlsx) can be imported to the Multi-File XF Report Generator.
- XFp – A maximum of 10 Excel files (*.xlsx) can be imported to the Multi-File XF Report Generator.

**Can I import Excel files generated on two different XF analyzers?**

No. Assay result data must be generated by the **same** Agilent Seahorse XF, XFe or XFp Analyzer – combining data from different analyzers is not supported at this time (example: XF96 and XFe96).

**Can I use the Excel file exported from the XFp, XF96, or XF24 Analyzer?**

Assay result data must be first exported from Wave 2.3 (Desktop or Controller) to an Excel file (*.xlsx), then imported to the Multi-File XF Report Generator. 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.

**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.

**If you receive an error message about Instrument Protocol (XFe96; XFe24; XF96; XF24 only)**

Errors upon data import are likely caused by a two reasons: mismatching Instrument Protocols (see Best Practices), or a c*ustom cycle* in one or more of the imported file Instrument Protocols. 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 is not supported in Report Generator analysis. Please contact Seahorse Technical Support for any additional questions regarding *Custom Cycles*.

**Where is my ECAR data?**

ECAR (Extracellular Acidification Rate) data for each *Group Collection* is calculated and displayed at the bottom of the *Measures Sheet* tab.

# Feedback

Feedback for the Report Generator and other software products is always encouraged and appreciated. Please direct any questions, concerns or suggestions to Seahorse Technical Support.

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# 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.

**Group Collection**

The collection of individual replicate groups from multiple Excel assigned to a single group. Each *Group Collection* must have at least 2 assay groups assigned to it from different Excel files.

**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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