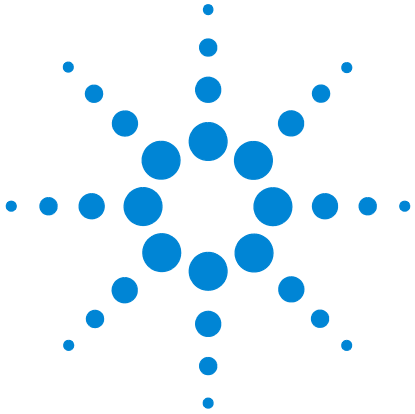

Agilent Seahorse XF Glycolytic Rate Assay Multi-File Report Generator

User Guide

Agilent Technologies

Notices

Manual Part Number

S7888-10009 (Rev A)

Edition

First edition, September 2017

Printed in USA

Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610 USA

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Contents

1 Introduction

Introduction

Agilent Seahorse XF data analysis and interpretation - accelerated. The Agilent Seahorse Multi-File XF Glycolytic Rate Assay Report Generator is the recommended software tool for analysis of replicate XF Glycolytic Rate Assay result data.

The Multi-File XF Glycolytic Rate Assay Report Generator is a Microsoft Excel Macro that automatically converts experimentally-derived OCR and ECAR data into Glycolytic Proton Efflux Rate (glycoPER), reported in pmol H^+/min (Table 1). More information on the relationship between PER and glycoPER can be found in the White Paper: Improving quantification of cellular glycolytic rate using Seahorse XF technology. More information on the conversion of Extracellular Acidification Rate (ECAR) data to Proton Efflux Rate (PER) data can be found in the Quick Reference Guide: Calculating Proton Efflux Rate (PER) Data.

Figures and data tables in the report generator can be easily transferred to other software programs for additional graphing or statistical analysis. The Multi-File XF Glycolytic Rate Assay Report Generator supports assay result data generated by Agilent Seahorse XFe96, XFe24, XFp and XF96 Analyzers. Wave 2.4 software is required to use the Multi-File XF Report Generator, earlier versions of Wave software are not compatible.

Table 1 Agilent Seahorse XF Glycolytic Rate Assay parameter equations

Parameter name	Parameter equation
Basal Glycolysis	Last glycoPER measurement before first injection.
Basal Proton Efflux Rate (PER)	Last PER measurement before first injection.
% PER from Glycolysis (Basal)	(Basal Glycolysis)/(Basil PER) x 100%
Compensatory Glycolysis	Maximum glycoPER measurement after Rot/AA injection.
mitoOCR/glycoPER (Basal)	[(Last OCR measurement before first injection) - (Minimum OCR after Rot/AA injection)]/(Basal Glycolysis)
Post 2-DG acidification	Minimum glycoPER measurement after 2-DG injection.

NOTE Definitions of PER and glycoPER can be found in the Agilent Seahorse XF Glycolytic Rate Assay Kit User Guide. Additional parameters are reported for an induced XF Glycolytic Rate Assay in "Induced Assays" on page 20.

Parameter Equations: XF Glycolytic Rate Assay

The XF Glycolytic Rate Assay provides accurate measurements of glycolytic rates for basal conditions and compensatory glycolysis following mitochondrial inhibition (Figure 1). The calculated glycolytic rates accounts for contribution of CO_2 to extracellular acidification derived from mitochondrial/TCA cycle activity, and are directly comparable to lactate assays.

Parameter calculations in the XF Glycolytic Rate Assay Report Generator are outlined in Table 1 on page 6. 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.

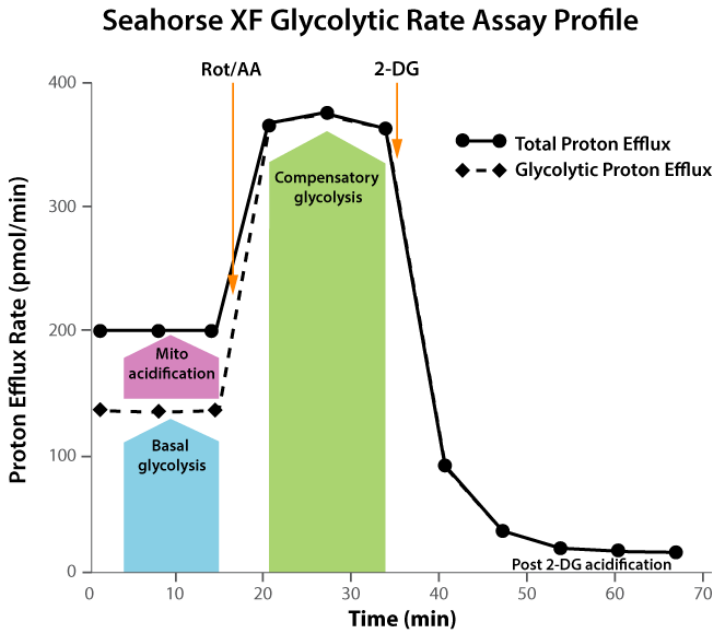

Figure 1 Agilent Seahorse XF Glycolytic Rate Assay parameters and kinetic profile.

Multi-File XF Report Generator Overview

The Multi-File XF Report Generator automatically calculates assay parameters of the XF Glycolytic Rate Assay (Table 1 on page 6) 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 report generator does not perform any statistical analysis (ANOVA; t-test, and so forth) on the imported data. More information can be found in the GraphPad Statistics Guide (H.J. Motulsky).

Example calculation workflow

Figure 2 on page 9 shows two groups, control and treatment, measured in three, independent replicate experiments:

1. Create a group collection, and assign each replicate control group from each experiment.
2. Calculate assay parameters first for individual wells within each replicate control group assigned to the group collection.
3. Calculate the average parameter value for each replicate control group.
4. Calculate assay parameters for the control group collection as the average of the average parameter value for each replicate control group in the group collection.

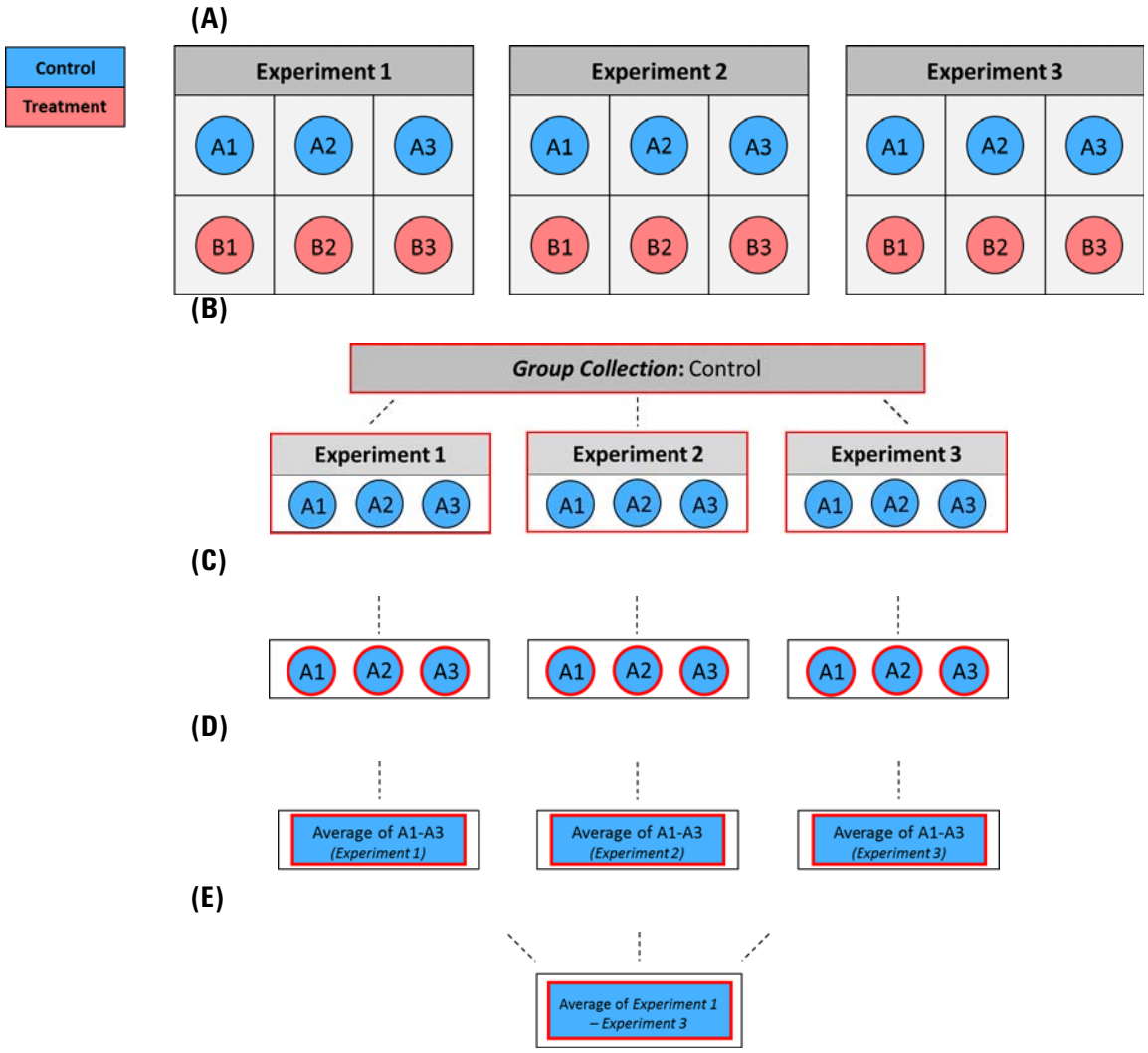

Figure 2 (A) Two groups, control and treatment, measured in three independent experiments.
(B) The control group from each experiment is assigned to a group collection.
(C) Assay parameters are calculated within each well (A1, A2, A3) for each replicate control group.
(D) The average of the individual well parameter values is calculated for each replicate control group.
(E) The final reported assay parameters represent the average ±S.E.M. of the average parameter value from each replicate control group in the group collection.

The Multi-File XF Glycolytic Rate Assay Report Generator displays result data and other assay-related information on 4-5 tabs:

- **Summary Printout: A** one-page Summary Report of the imported XF Glycolytic Rate Assay result files for the configured group collections.
- **Normalize:** Plate maps of normalization values applied to each imported result file. See the Wave User Guide for more information.
- **Measures sheet:** Data tables of average assay parameter values and buffer factor per file, rate data for glycoPER, PER, ECAR and OCR, and corresponding kinetic graphs.
- **Assay parameter per well:** Data table of assay parameters per well for each group collection.
- P**roject information:** Software license terms, plate and cartridge details, and plate map layout for each imported file.

Multi-File XF Report Generator: Technical Requirements and 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 XFe96, XFe24, XFp, or XF96 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 2.4 software that will be used to perform each replicate XF Glycolytic Rate Assay. Using the same assay template for each experiment ensures consistent group naming, instrument protocol, and other important assay-related information.
- Apply normalization data in Wave 2.4 software before exporting to Excel. This allows for analysis of normalized and non-normalized rate data in the Multi-File XF Report Generator.
- Assign at least three wells for each group in your assay template file.
- It is recommended to measure the same experimental groups/conditions in each replicate experiment.
- Inspect and analyze the kinetic graph - review the kinetic graphs for each independent assay result file in Wave 2.4 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 or ECAR. Optimal density may be obtained either through empirical assay or recommended values found in the XF Cell Reference Database, XF Publications Database, or XF Assay Template/Guides.
- Maintain consistent cell culture or subculture of cells, including but not limited to passage number, subculture density, serum lot, and so forth for each replicate experiment.
- Maintain a consistent time of cell growth after seeding, and a consistent time of treatment or intervention for each replicate experiment.
- Ensure the cells maintain a consistent baseline rate in absence of any treatments or injected compounds. The baseline rate is defined as the rate measurements before injection from Port A.
- Well-to-well consistency within experimental groups should be reviewed in Wave 2.4 software (or later) for consistent cell seeding technique. Use the **Display** toggle (above the kinetic graph) to display kinetic rate data in **Well** mode to assist in evaluating well-to-well variability and outliers in a group.

2 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 Multi-File XF Glycolytic Rate Assay Report Generator is a Microsoft Excel Macro-Enabled Template file, and is compatible with Microsoft Excel versions 2010, 2013, and 2016 for Windows PCs, and Microsoft Excel for Mac versions 2011 and 2016. To use this report generator, Excel must be configured to allow macros to run. To enable macros once, double-click the Multi-File XF Glycolytic Rate Assay Report Generator.xltm file, then click **Enable Editing** and **Enable Content** (yellow information bar) if prompted (Figure 3).

Figure 3 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**.

Analyze Data in the Multi-File XF Report Generator

Compile and analyze multiple data files in the Multi-File XF Report Generator following the five steps below:

Step 1 - Download the Multi-File XF Glycolytic Rate Assay Report Generator:

1. Using any personal computer with internet access, visit the report generator webpage on the Agilent website, and register to download the XF Glycolytic Rate Assay Report Generators.
2. Once the download is complete, unzip the compressed folder (right-click the folder and click **Extract all...**).
3. The download includes both the Single-File and Multi-File XF Glycolytic Rate Assay Report Generators.

Step 2 - Export assay result data as Excel Workbook files (.xlsx) using Wave software:

1. Using Wave 2.4, open an assay result file and click Export.

NOTE

If using more than one assay media, select only those groups and background wells on the plate map that contain the same assay media before Excel export. Repeat the Excel export for assay groups and background wells containing assay media 2.

2. Select **Microsoft Excel,** and click **Save**.
3. Repeat for each assay result file that will be analyzed with 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 XF Glycolytic Rate Assay Report Generator.xltm** file.
2. Click **Enable Editing** and **Enable Content** after opening the report generator if prompted (Figure 3 on page 14).
3. Click **Load New Data Files**.
4. Locate the Excel files exported from Wave 2.4 software.
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 4) 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.

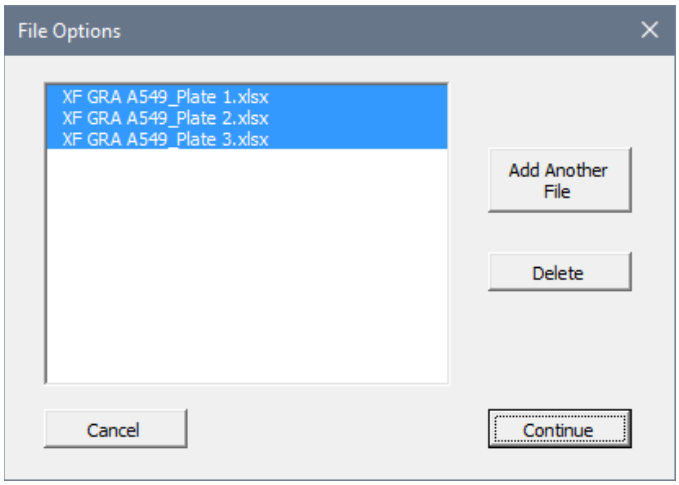

Figure 4 File Options window displaying imported Excel files for analysis in the report generator.

Step 4 - Configure Group Collections:

The **Configure Group Collections** window displays the list of groups for each imported Excel file as entered in the assay template file (Figure 5 on page 17).

NOTE

It is critical that the correct replicate group (or condition) from each Excel file is assigned to the same group collection. Parameter calculations are based on the individual group data assigned to each group collection. See "Multi-File XF Report Generator: Technical Requirements and Best Practices" on page 11 for more information.

1 Create a group collection by assigning the replicate group/condition from each imported Excel file. The example in Figure 5 on page 17 shows a group collection comprised of three replicate groups - "control" - measured in three 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.

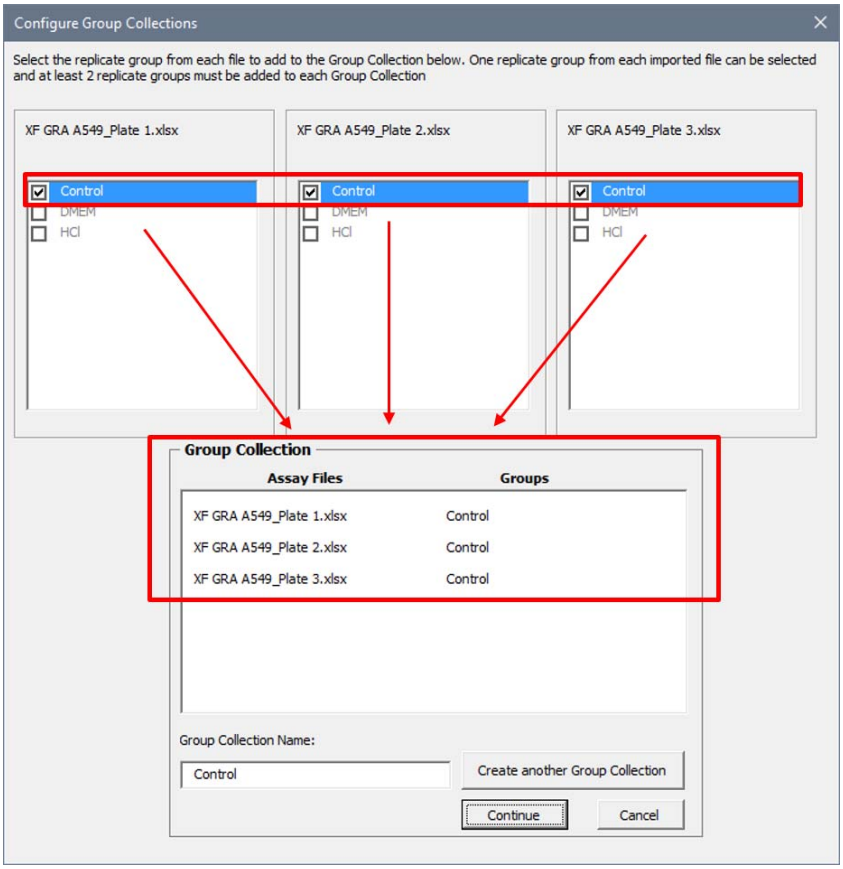

Figure 5 Configure Group Collections window. Check the box next to each replicate group or conditions within the imported Excel file. Select groups and the corresponding Excel file are displayed at the bottom of the window.

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 6 on page 18). If your assay has more than two injections, see "Induced Assays" on page 20.

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.

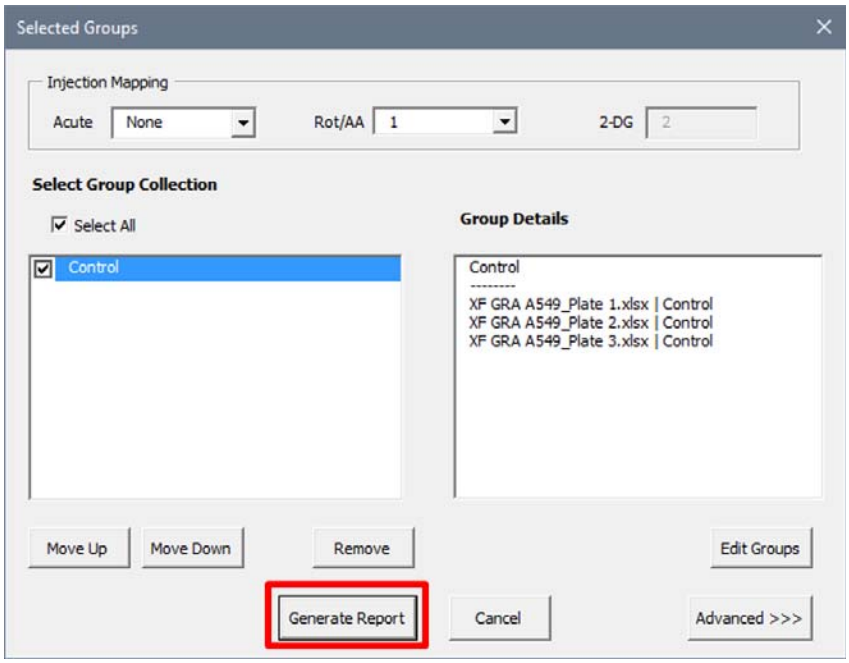

Figure 6 List of configured group collections and details of each selected group collection within the **Group Details** box. Use the **Move Up/Move Down** buttons to modify the order of group collections when viewing results in the Multi-File XF Report Generator. Click **Generate Report** to create a summary report of the selected group collections.

Advanced Options

Access Advanced Options (Figure 7) by clicking **Advanced** on the **Display Options** window. Advanced Options displays the CO_2 Contribution Factor (CCF) per group. The CO_2 Contribution Factor should only be adjusted after assessing results from your first XF Glycolytic Rate Assay. See the *CO_2 Contribution Factor Protocol User Guide* for more information.

Buffer factor values cannot be modified in the Multi-File XF Report Generator, only in Wave 2.4 software before exporting data. Buffer factor values are exported to Excel per group and automatically imported to the Multi-File XF Report Generator. To view the buffer factor values used to calculate assay parameters, see the **Measures Sheet** tab (after generating a summary report).

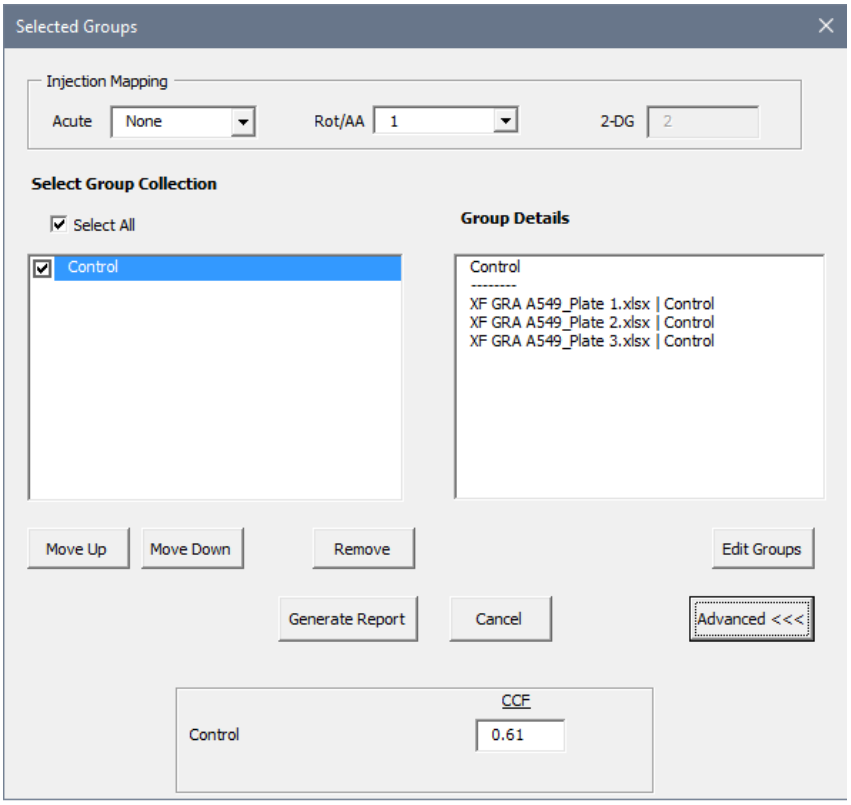

Figure 7 Editable fields for CO_2 Contribution Factor (C.C.F.) seen in Advanced Options.

Induced Assays

The XF Glycolytic Rate Assay with an acute injection is called an induced assay. An acute injection is an injection that occurs after the baseline measurements but before the Rotenone/Antimycin A (Rot/AA) injection. The **XF Glycolytic Rate Assay (Induced Assay)** assay template is specifically designed for this type of assay. For custom assays, the acute injection must be manually added to the Instrument Protocol prior to starting the assay.

- The acute injection must be injected before Rot/AA from Port A on the cartridge.
- The report generator automatically displays an additional field on the Display Options window called Injection Mapping (Figure 8). The acute injection must be identified using the **Injection Mapping** drop-down menu before generating a Summary Report. If **None** is selected, then the position of the Rot/AA injection must be assigned.
- The report generator will report additional assay parameters for an induced assay. Parameter names and equations are displayed in Table 2 on page 21.

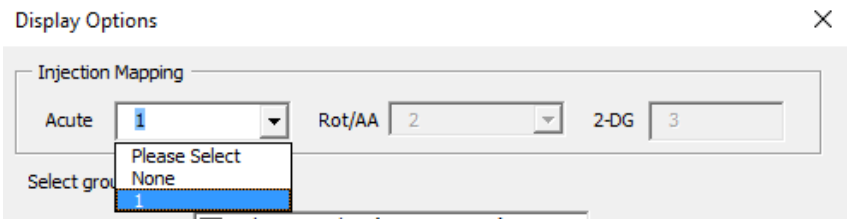

Figure 8 Injection Mapping drop-down menu is displayed after importing an assay result file with more than two injections. After identifying the acute injection, the report generator automatically assigns the Rot/AA and 2-DG injections sequentially.

Table 2 Agilent Seahorse XF Glycolytic Rate Assay (Induced) parameter equations

Parameter name	Parameter equation
Induced Glycolysis	Average (Avg.) of the glycoPER measurements after the induced assay injection and before Rot/AA injection.
Induced PER	Avg. of the PER measurements after the induced assay injection and before next injection.
% PER from Glycolysis (Induced)	(Induced Glycolysis)/(Induced PER) x 100%
Induced mitoOCR*	(Avg. of the OCR measurements after induced injection and before next injection) - (Minimum OCR after Rot/AA injection)
mitoOCR / glycoPER (Induced)	(Induced mitoOCR)/(Induced Glycolysis)

* Induced mitoOCR is not reported separately in the XF Glycolytic Rate Assay Report Generator.

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:

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

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 (Figure 9).

Group collection	File number	Avg. Basal Glycolysis (glycoPER)
'Control'	Control group (file 1)	231 pmol/min
	Control group (file 2)	215 pmol/min
	Control group (file 3)	242 pmol/min

$$\text{Basal Glycolysis (glycoPER) S.E.M.} = \frac{\text{Standard Dev. (231,215,242)}}{\sqrt{(3)}} = \frac{13.58}{1.73} = \pm 7.84 =$$

Figure 9 Example S.E.M. calculation for Basal Glycolysis (glycoPER) for the 'control' group collection.

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 10) and any assay wells or groups that have been excluded from the Excel export (Figure 10).

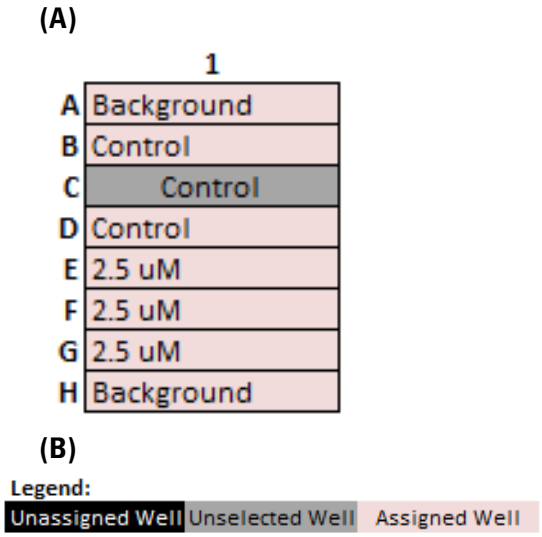

Figure 10 (A) XFp Plate Map on the Project Information tab. Assay well C has been turned off in Wave 2.4 prior to Excel export, therefore the control group parameter calculations are based on assay wells B and D only.
(B) Formatting legend for the Plate Map(s) to indicate the assay wells that are Assigned, Unselected, and Unassigned for parameter calculations.

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.

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 2.4 (Figure 11a) 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 2.4 (Figure 11b). 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 11c).

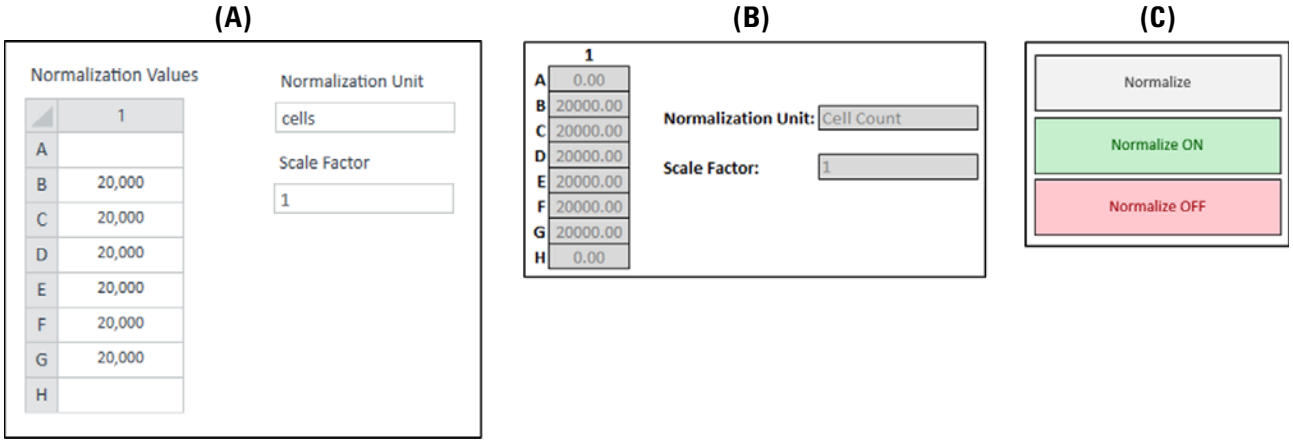

Figure 11 (A) Shows an example normalization plate map in Wave 2.4 for the XFp Analyzer.
(B) Shows the same normalization plate map values displayed in the report generator.
(C) Shows the **Normalize** button on the **Summary Printout** tab.
Data exported from Wave 2.4 without normalized rate data will 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 2.4 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 2.4 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.

3 Frequently Asked Questions

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

  Parameter equations are described in Table 1 on page 6 and Table 2 on page 21.

- **Incompatible elements with the XF Glycolytic Rate Assay**

  The following items are not supported with the XF Glycolytic Rate Assay:

  - Sensor cartridges for the XF24 and XF24-3 Analyzer.
  - Spheroid, Islet, and V28 plate types.

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

  Outlier assay wells must be turned **OFF** or reassigned in Wave 2.4 prior to Excel export. See "Exclude Outliers/Groups from Analysis" on page 23 or the Wave User Guide for more information.

- **I need to change Buffer Factor for a group, where can I find this?**

  Buffer Factor values are automatically imported from each Excel File per group. Modifying the Buffer Factor in the Multi-File XF Report Generator is not supported at this time. To change the Buffer Factor value for a group, open the assay result file in Wave 2.4 software, change the **Buffer Factor** value of the assay media and the background wells, and re-export the data file to Excel.

- **Can I use the Excel file exported from the XFp, XF96 Analyzer, or an earlier version of Wave software?**

  This Multi-File XF Report Generator supports import and analysis of Excel files exported from Wave 2.4 (Desktop or Controller) or later. Excel files exported from the XFp Analyzer, XF96 Analyzer, XF Reader software, or earlier versions of Wave software are not supported.

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

  Custom Cycles are not part of the standardized assay template for the XF Glycolytic Rate Assay and not supported in Report Generator analysis. A Custom Cycle refers to an additional 'Mix' or 'Wait' command step in the Instrument Protocol of an assay. Please contact Agilent 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) ECAR data is used for parameter calculations, parameter calculations using baseline rate data (%) is not supported at this time.

- **Warning message(s) when the % PER from Glycolysis is less than 50% and less than 10%**

  When the % of PER from Glycolysis for a selected group is less than 50% or less than 10%, the Report Generator will display a warning message in the legend on the Summary Printout tab (below the Normalize button). For more information, see “Advanced Options” on page 19.

Feedback

Feedback for the Report Generator or other products is always encouraged. Please direct any questions, concerns or suggestions to Agilent Seahorse Technical Support at: AgilentSeahorse.support@agilent.com

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Printed in USA, September 2017
First edition

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S7888-10009 (Rev A)