



Agilent Seahorse  
**Wave Pro & XF Pro Controller**  
For use with Agilent Seahorse XF Pro Analyzers  
**Software User Guide**

# Notices

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# Introduction

Agilent Seahorse Wave Pro is software used for creating assay template files for the Agilent Seahorse XF Pro and XF HS Mini Analyzers, as well as viewing & analyzing result data from these analyzers. Wave Pro software can be installed on your personal computer (Microsoft Windows OS only) as well as the computer connected to the XF Pro Analyzer, called the XF Pro Controller.

Agilent Seahorse XF Pro Controller is software used for operating the XF Pro Analyzer (only). This software can also be used for creating assay template files for the XF Pro Analyzer. XF Pro Controller software can be installed on the XF Pro Controller (computer connected to the XF Pro Analyzer) only. This software cannot be installed on your personal computer.

This user guide provides detailed information on various functions in both software applications, Wave Pro and XF Pro Controller.

# Quick Start

The typical software workflow can be outlined in three steps:

- Design - create an assay template file
- Acquire - perform an XF assay & acquire data
- Analyze - open & review results

## Create an assay template file

The first step in the XF software workflow is to create an assay template file. An assay template file is comprised of information about your experiment (e.g., conditions to test, assignment of groups on plate map, etc.) as well as the instrument protocol, the series of commands performed during an assay, the timing of when these commands occur, and the duration. There are several pre-populated assay template files provided in Wave Pro & XF Pro software for Agilent Seahorse XF Assay Kits (e.g., XF Real-Time ATP Rate Assay). It is recommended to start with a pre-populated Seahorse assay template and customize as needed.

See "Create/Edit Assay Templates" on page 14 for more information on creating/customizing assay templates.

## Perform XF assay, acquire data

Once your assay template file is ready you can run your XF assay. If you created the template file on your personal computer using Wave Pro software, then transfer the template to the XF Pro Analyzer (using a USB flash drive or active shared network drive). If you created the assay template on the XF Pro Controller (computer), no file transfer is needed, you can begin your assay when ready.

## Open & review results

After the assay is finished, you can open & review data using Wave Pro software (on the XF Pro Controller), or you can transfer the data file to your personal computer and analyze results using Wave Pro or Agilent Seahorse Analytics (recommended).

# Wave Pro & XF Pro Home View

## **Templates**

When you open Wave Pro software or XF Pro Controller software, you will see the Templates view. View and organize your assay templates from the Templates view. Use the Create, Export, Import, and Duplicate buttons to manage your templates list.

## **Results**

View, open, and manage your assay result files from the Results view. Please note the Results view is available in Wave Pro software only. The Results view is not displayed in XF Pro Controller software.

## **Diagnostics**

Perform system and consumable diagnostic check tests, perform instrument QC tests, and review previous QC results from the Diagnostics view. Please note the Diagnostics view is available in XF Pro Controller software only.

## **Catalog**

Use the Catalog to save & edit frequently used group definitions (Compounds, Pretreatments, Media, and Cells) that are frequently used for template creation and modification. Wave Pro & XF Pro software provide a prepopulated Catalog containing a variety of components available in Seahorse XF assay kits & reagents, assay medium, etc.

## **Help**

Agilent Cell Analysis Technical Support contact info, software version and auto-compile feature for System Files.

## **Options**

Configure local file directories (for assay templates and the catalog), image database path, instrument protocol default values. Additional options are provided in XF Pro Controller software to configure the Agilent Seahorse XF Discovery license trial period or to claim your XF Discovery license.

## 1 Introduction

### Wave Pro & XF Pro Home View

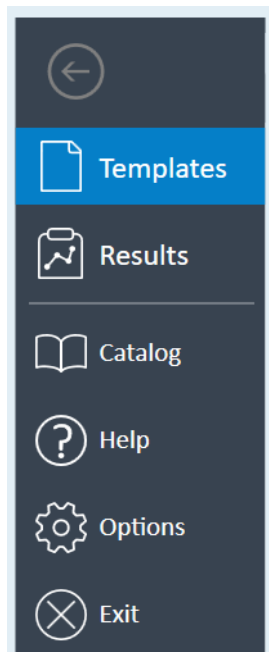


Figure 1. Wave Pro Home Views

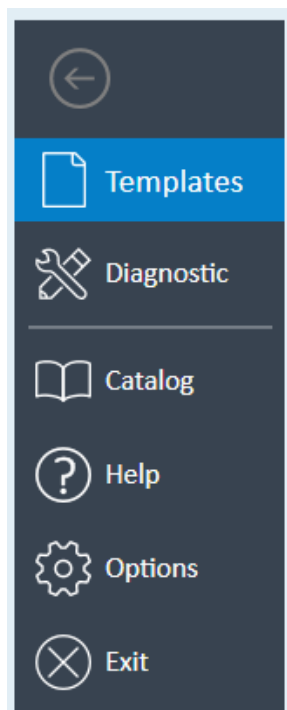


Figure 2. XF Pro Controller Views

# Wave Pro & XF Pro: Templates View

The Templates view displays all assay template files saved in the Template Directory location on the XF Pro Controller (using XF Pro Controller software and/or Wave Pro software) and on your personal computer (using Wave Pro software only). (Figure 2.)

An assay template file (file extension ".xsyt") contains the necessary information to run an assay on the XF Pro Analyzer and XF HS Mini Analyzer.

An assay template file contains the following information: group definitions, plate map layout, instrument protocol, and more. An assay template can be reused as many times as necessary, shared with other users, and can be duplicated & customized as a new assay template for another assay on the XF Pro or XF HS Mini Analyzer.

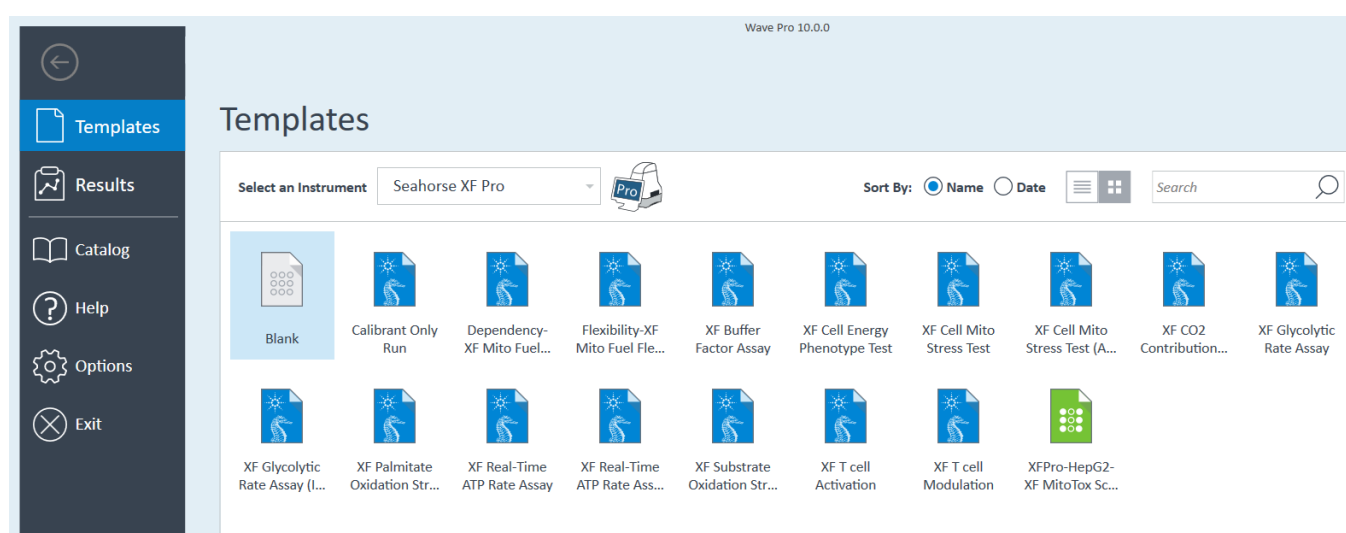


Figure 3. Wave Home > Templates view

Assay templates are color coded based on the type of assay template. (See [Table 1](#))

**Table 1** Assay templates

Template type	Template color
Agilent Seahorse	Blue
Blank	White
User-customized	Green

Wave Pro software & XF Pro software automatically install several Seahorse template files containing specific assay-related information for each Seahorse XF Assay Kit. The blank and Seahorse templates are not editable. Changes made to these templates will always be saved as a new assay template (green icon). (See [Figure 4](#))

## 1 Introduction

### Wave Pro & XF Pro: Templates View

Blank  
template



Blank

Agilent Seahorse  
default template



XF Cell Energy  
Phenotype Test

User-customized  
template



Ben's New  
Template

Figure 4. Types of assay template files



## Wave Pro (only): Results view

The Results view provides access to your assay result data files and is available in Wave Pro software only. This view shows recently opened assay result files, the corresponding file directory where each assay result file is located, and Favorite Places.

(See Figure 4.) Opening a new assay result file displays the default analysis view - the Quick View. After modifying and saving an assay result file, Wave automatically displays the last modified analysis view the next time the assay result file is opened.

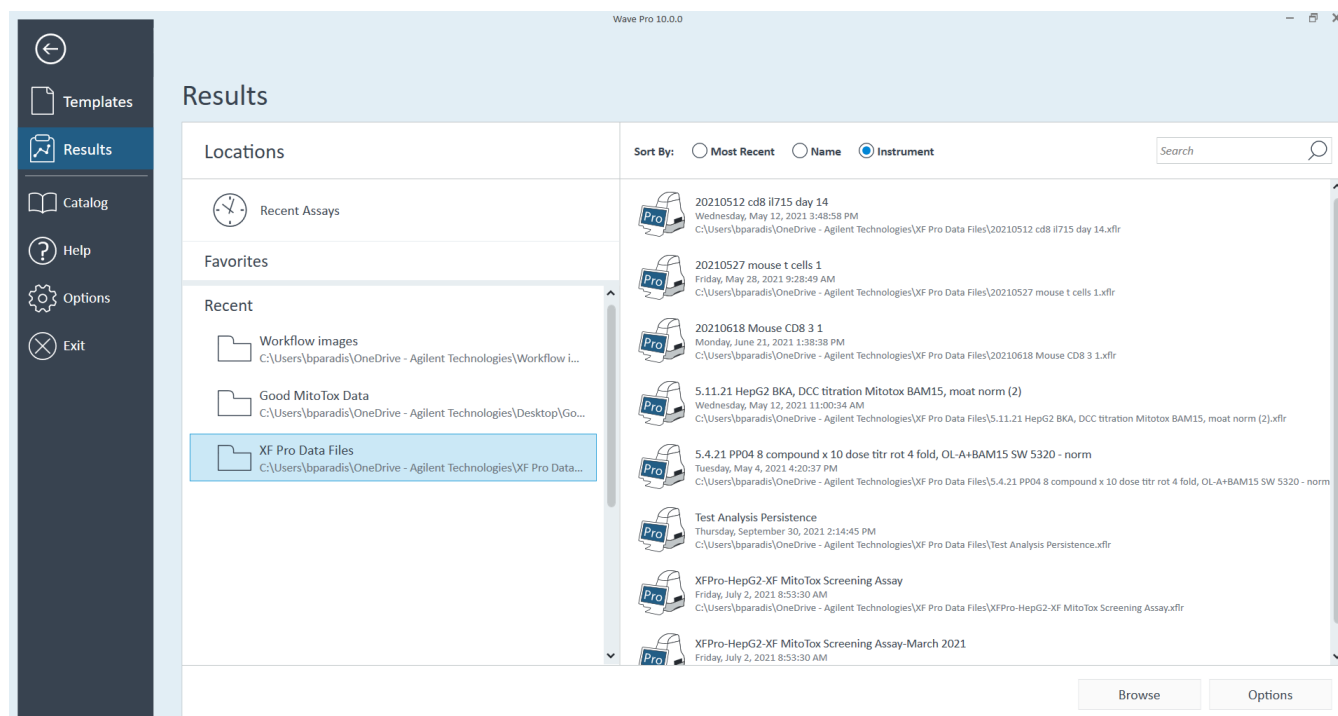


Figure 5. Wave Home > Results view

## Design XF Assays with Wave

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## Create/Edit Assay Templates

To create a new assay template or modify an existing template:

- 1 Open **Wave Pro** or **XF Pro Controller** software. **Templates** is the default (first) view when you open the software.
- 2 Select the **Blank** template, a **Seahorse** template, or a **user-customized** template and click **Open** (double-click the template icon).
- 3 Add, remove, or modify group definitions, and create new assay groups. (See **“Step 1: Group Definitions”** on page 16.)
- 4 Assign groups to the Plate Map. (See **“Step 2: Plate Map”** on page 33.)
- 5 Review or modify Instrument Protocol. (See **“Step 3: Instrument Protocol”** on page 34.)
- 6 Save the template, transfer it to XF Pro or XF HS Mini Analyzer. (See **“Saving Assay Templates”** on page 49.)
- 7 Run the assay. (See **“Step 4: Run Assay”** on page 37.)

## Step 1: Group Definitions

There are four types of group definitions you can add to an assay template:

- Injection Strategies
- Pretreatments
- Assay media
- Cell type

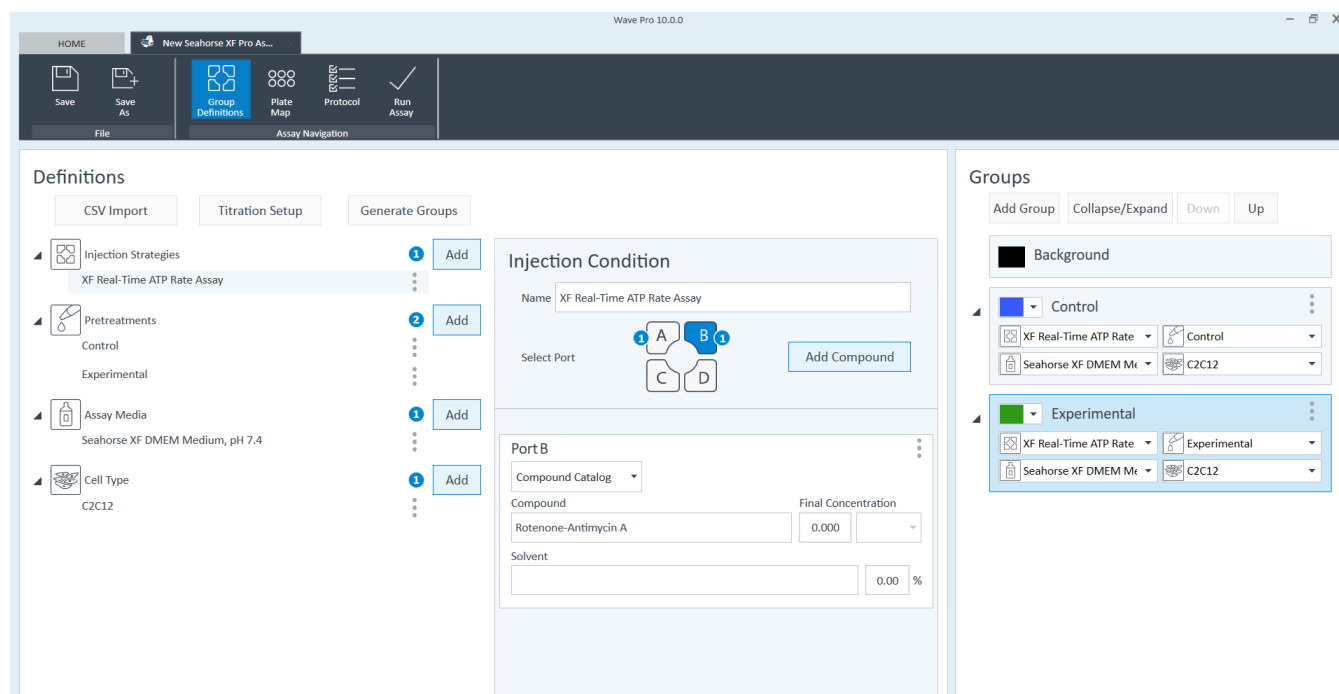


Figure 6. Group definitions

### Injection strategies

Injection strategies describe the contents of up to four injections (one for each of the four ports: A, B, C, and D). More than one injection strategy can be performed in an assay. For example, one injection strategy can be defined for the XF Real-Time ATP Rate assay, and one for the XF Cell Mito Stress Test, on the same plate.

To add one or more injection strategies:

- 1 Click **Add** next to **Injection Strategies**. (See [Figure 6](#).)
- 2 Enter a name for the injection strategy if desired. The default name when adding new injection strategies is **Inj. Strategy #** (# corresponds to how many injection strategies have already been added).

## 2 Design XF Assays with Wave Pretreatments

- Next, specify the contents of each injection starting with Port A. Click **Add Compound**, and type in a compound name (for example oligomycin), or use the **Compound Catalog** drop-down menu to select a compound from the catalog. (See [Figure 7.](#))

The figure shows two identical 'Injection Condition' panels side-by-side. Each panel has a 'Name' field set to 'XF Real-Time ATP Rate Assay'. Below the name is a 'Select Port' diagram with four ports: A (top-left, blue), B (top-right, blue), C (bottom-left, white), and D (bottom-right, white). To the right of the diagram is an 'Add Compound' button. Below the diagram, there are two sections: 'Port A' and 'Port B'. Each section has a 'Compound Catalog' dropdown, a 'Compound' text field, a 'Final Concentration' text field, and a 'Solvent' text field. In the 'Port A' section, 'oligomycin' is entered in the 'Compound' field and '0.000' is entered in the 'Final Concentration' field. In the 'Port B' section, 'Rotenone-Antimycin A' is entered in the 'Compound' field and '0.000' is entered in the 'Final Concentration' field.

Figure 7. Assign injection ports

- Repeat [step 3](#) for injections from ports B, C, and D.

[Figure 8](#) shows one injection strategy for the XF Real-Time ATP Rate Assay:



Figure 8. The XF Real-Time ATP Rate Assay injection strategy

## Pretreatments

Pretreatments describe a treatment the cells have received prior to performing the assay, such as a genetic manipulation or prolonged exposure to compounds.

To add one or more Pretreatments:

- Click **Add** next to **Pretreatments**. (See [Figure 9.](#))



Figure 9. Pretreatment conditions

## 2 Design XF Assays with Wave

### Assay media

- 2 Enter a name for the pretreatment or use the Pretreatment drop-down menu to select a pretreatment from the catalog. The default name when adding new pretreatments is **Pretreatment #** (# corresponds to how many pretreatments have already been added). **Figure 9** displays two Pretreatment conditions, **Control** and **Experimental**.
- 3 If desired, specify the type of pretreatment using the **Description** field.

## Assay media

Assay Media describes one or more assay mediums used in the assay.

Agilent Seahorse XF assay media are formulated based on common culture media DMEM or RPMI, and are optimized for use in XF assays. Unlike typical cell growth or culture media, XF assay media are free of bicarbonate, low in buffer capacity, and contain low or no phenol red, which is important for achieving robust results. Seahorse XF media with pH 7.4 is also available, simplifying the workflow, saving time for assay preparation, and ensuring consistent results across experiments.

Record the medium and supplements for each medium used in the assay:

To add one or more Assay Medium:

- 1 Click **Add** next to **Assay Media**. (See **Figure 10**.)

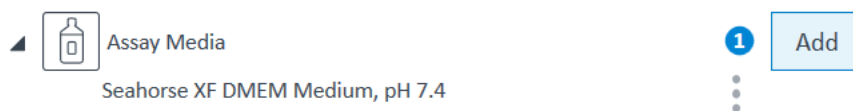


Figure 10. Assay Media

- 2 Enter a name for the assay media, or use the Media drop-down menu to select a media from the catalog. The default name when adding a new assay media is **Assay Media #** (# corresponds to how many assay media have already been added). (See **Figure 10**.)
- 3 Enter additional details if desired for: Source, Supplements, Prepared By, and so on.

## Cell type

Cell Type describes the biological material/samples used, including information about the cell line, seeding density, and passage number.

To define one or more Cell Types:

- 1 Click Add next to Cell Type. (See **Figure 11**.)



Figure 11. Cell Type

- 2 Enter a name for the cell type or use the Cell Line drop-down menu to select a cell type from the catalog. The default name when adding a new cell type is **Cell Type #** (# corresponds to how many cell types have already been added).
- 3 If desired, enter additional details for: Seeding Density, Lot, Source, and Passage number.

## Duplicate and Delete

The three dots next to each group definition enable two functions: Delete the group definition entry and Duplicate the group definition entry. (See [Figure 12.](#))



Figure 12. Duplicate and Delete function toggle

## Automatically generate groups

After creating the individual Group Definitions, the next step is to create assay groups and assign the various definitions to each group based on your experimental design. This can be done automatically by clicking the **Generate Groups** button. This function uses the independent Group Definitions to calculate the number of groups, assuming every possible combination of independent conditions. An example of the combinatorial logic using the Group Definitions below shows how this function results in a total of eight unique groups ( $1 * 2 * 1 * 1 = 2$ ):

- Injection Strategies: 1 (XF Real-Time ATP Rate Assay)
- Pretreatments = 2 (Control and Experimental)
- Assay Media = 1 (Seahorse XF DMEM Medium, pH 7.4)

## 2 Design XF Assays with Wave

### Manually generate groups

- Cell Type = 1 (C2C12)

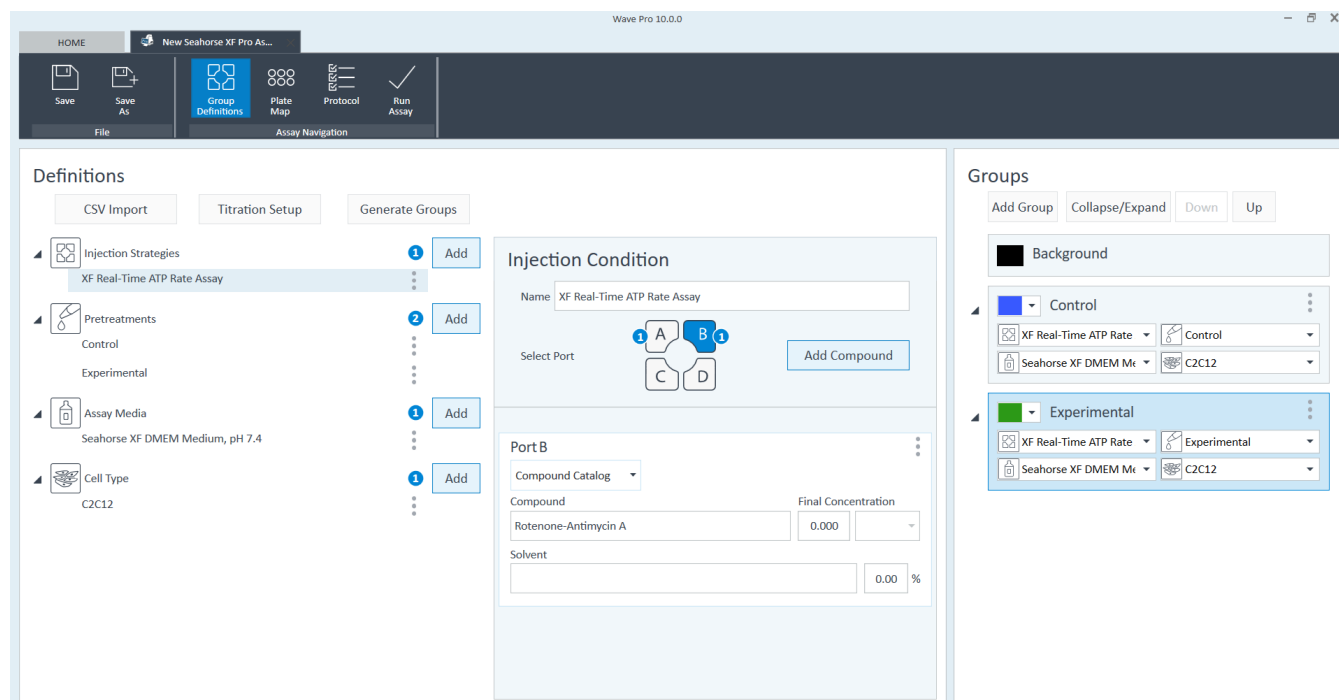


Figure 13. Automatically generate groups

## Manually generate groups

If desired, you can create groups manually:

- 1 Click Add Group to create Group 1. (See [Figure 14](#).)

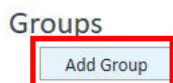


Figure 14. Add groups

- 2 Use the drop-down menu for each group definition to assign the appropriate conditions ([Figure 15](#).)

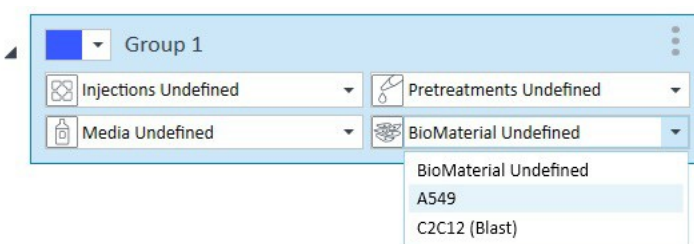


Figure 15. Drop-down menus for group definitions

- 3 Repeat for each manually generated group.



## 2 Design XF Assays with Wave

### Import group definitions and plate map layout using the CSV import feature

To change the name of the group, double-click the **Group 1** text, or right-click and select **Edit**. To change the color of the group, use the drop-down color menu next to the group name. After adding Group Definitions and generating groups, assign groups to the Plate Map. (See “**Step 2: Plate Map**” on page 33..)

## Import group definitions and plate map layout using the CSV import feature

The CSV import feature, available in both Wave Pro and XF Pro software, allows you to quickly create detailed assay template files (e.g., for a compound screen) by importing an Excel spreadsheet - a specially formatted Excel file saved in the CSV file format - containing the desired group definitions, names, plate map assignments, and more.

To use the CSV Import feature:

- 1 Launch Wave Pro or XF Pro software
- 2 Open the Blank template from the Templates view (**Figure 16**).

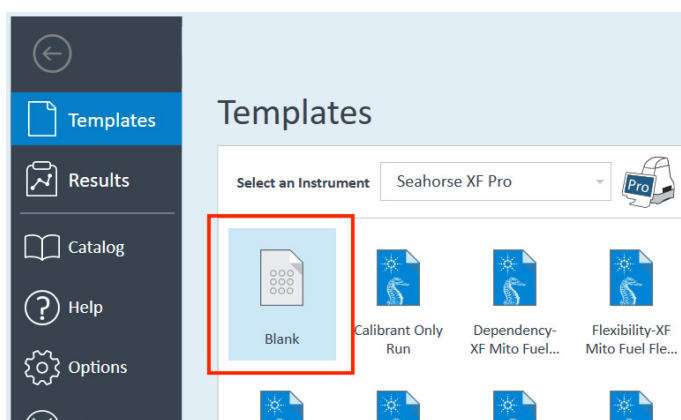


Figure 16. The Blank template pictured in the Templates view.

- 3 On the Group Definitions view, click **CSV Import** (**Figure 17**)

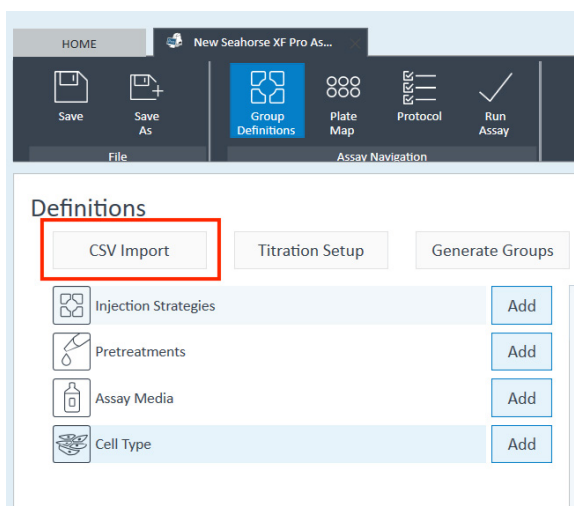


Figure 17. CSV Import button..

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

You are now on the CSV Import view (**Figure 18**).

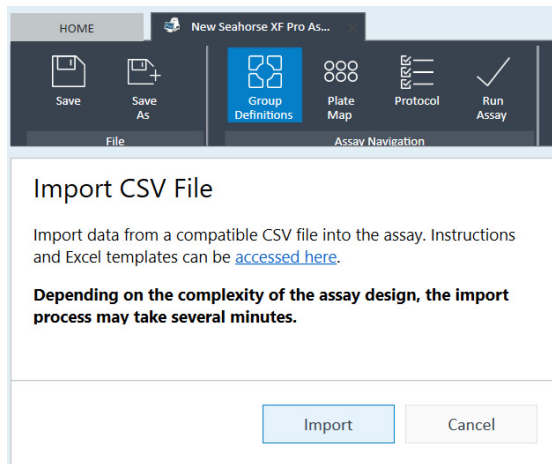


Figure 18. The CSV Import view.

If you have already created your Excel CSV file, then go to **step 13**.

OR

If you have NOT created your Excel CSV file, then you must create your CSV file using the file called Agilent-Seahorse-XF-Pro-Assay-Import-Template.xlsx.

- 4 Create a blank copy of the Agilent-Seahorse-XF-Pro-Assay-Import-Template.xlsx file from CSV Import view by clicking the link pictured in **Figure 19**.

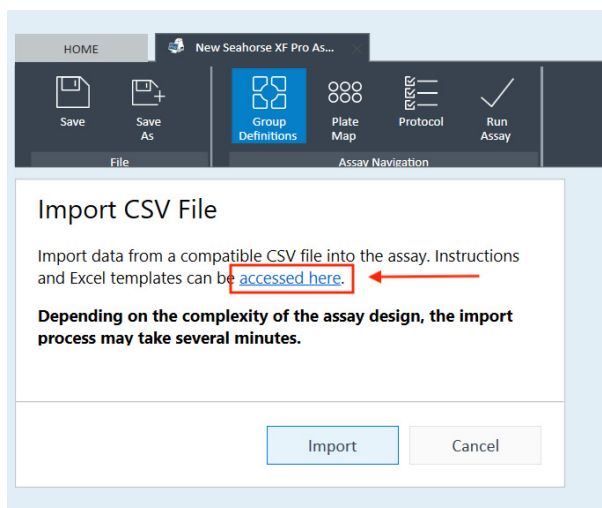


Figure 19. Create new copies of the Excel import template using the red outlined link.

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

- 5 Click **OK** on the **Files Found** dialog (Figure 20).

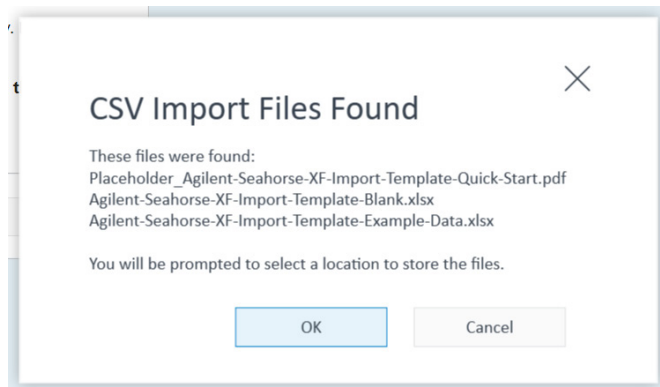


Figure 20. Files found dialog.

- 6 Select a file save location (Figure 21)

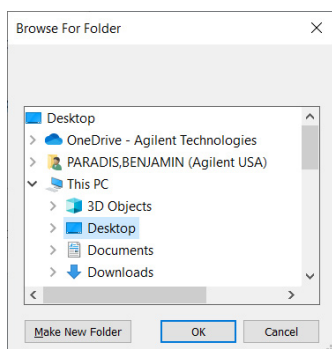


Figure 21. Choose a save location for the CSV Import files.

- 7 Open the file called: *Agilent-Seahorse-XF-Pro-Assay-Import-Template.xlsx*.
- 8 Enter a Project Name into cell A3 (Figure 22).

### NOTE

A Project Name is required to use the CSV import feature. If this cell is left blank, you will see an error (Figure 39).

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

	A	B	C
1		<b>Assay Information</b>	
2	Project Name	Principal Investigator	Plated By
3	New XF Project Name		
4		<b>Assay Groups</b>	
5	Group Name	Control Group Type	Well
6			A1
7			A2
8			A3
9			A4
10			A5
11			A6
12			A7
13			A8
14			A9

Figure 22. Enter a Project Name into cell A3. This field is required.

- 9 Designate the corner well positions A1 (row 6), A12 (row 17), H1 (row 90), and H12 (row 101) as Background group wells (Figure 23). If you have unassigned assay wells, do not leave these rows blank. Assay wells not assigned to a group should be designated as Background group wells prior to CSV import.

	A	B	C
1		<b>Assay Information</b>	
2	Project Name	Principal Investigator	Plated By
3	New XF Project Name		
4		<b>Assay Groups</b>	
5	Group Name	Control Group Type	Well
6	Background		A1
7			A2
8			A3
9			A4
10			A5
11			A6
12			A7
13			A8
14			A9
15			A10
16			A11
17	Background		A12

Figure 23. Two Background group assignments for wells A1 and A12.

- 10 If your experiment design requires control groups, you can assign special control group attributes to wells in your assay template. Please refer to the following list:

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

- Type Positive in column B for the appropriate rows/wells (**Figure 24**) to designate those wells as the Positive Control group in your assay template file (**Figure 25**).

1		Assay Information	
2	Project Name	Principal Investigator	Plated By
3	New XF Project Name		
4		Assay Groups	
5	Group Name	Control Group Type	Well
6	Background		A1
7	Positive Control Group Name	Positive	A2
8	Positive Control Group Name	Positive	A3
9	Positive Control Group Name	Positive	A4
10	Positive Control Group Name	Positive	A5
11	Positive Control Group Name	Positive	A6
12	Positive Control Group Name	Positive	A7
13	Positive Control Group Name	Positive	A8
14	Positive Control Group Name	Positive	A9
15	Positive Control Group Name	Positive	A10
16	Positive Control Group Name	Positive	A11
17	Background		A12
18			B1

Figure 24. Assay wells A2 to A11 (rows 7 to 16) are assigned the Positive control group type in the CSV Import file.









	1	2	3	4
A				
B				

Figure 25. A plus sign denotes assay wells assigned to the Positive control group type.

- Type Negative in column B for the appropriate rows/wells (**Figure 26**) to designate those wells as the Negative group in your assay template file (**Figure 27**).

1		Assay Information	
2	Project Name	Principal Investigator	Plated By
3	New XF Project Name		
4		Assay Groups	
5	Group Name	Control Group Type	Well
6	Background		A1
7	Negative Control Group Name	Negative	A2
8	Negative Control Group Name	Negative	A3
9	Negative Control Group Name	Negative	A4
10	Negative Control Group Name	Negative	A5
11	Negative Control Group Name	Negative	A6
12	Negative Control Group Name	Negative	A7
13	Negative Control Group Name	Negative	A8
14	Negative Control Group Name	Negative	A9
15	Negative Control Group Name	Negative	A10
16	Negative Control Group Name	Negative	A11
17	Background		A12
18			B1
19			B2

Figure 26. Assay wells A2 to A11 (rows 7 to 16) are assigned the Negative control group type in the CSV Import file.

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature









	1	2	3	4
A				
B				

Figure 27. A minus sign denotes assay wells assigned to the Negative control group type.

Type *Vehicle* in column B for the appropriate rows/wells (**Figure 28**) to designate those wells as the Vehicle group in your assay template file (**Figure 29**).

	A	B	C	
1	<b>Assay Information</b>			
2	Project Name	Principal Investigator	Plated By	Plated
3	New XF Project Name			
4	<b>Assay Groups</b>			
5	Group Name	Control Group Type	Well	Media
6	Background		A1	
7	Vehicle Group Name	Vehicle	A2	
8	Vehicle Group Name	Vehicle	A3	
9	Vehicle Group Name	Vehicle	A4	
10	Vehicle Group Name	Vehicle	A5	
11	Vehicle Group Name	Vehicle	A6	
12	Vehicle Group Name	Vehicle	A7	
13	Vehicle Group Name	Vehicle	A8	
14	Vehicle Group Name	Vehicle	A9	
15	Vehicle Group Name	Vehicle	A10	
16	Vehicle Group Name	Vehicle	A11	
17	Background		A12	
18			B1	
19			B2	
20			B3	
21			B4	

Figure 28. Assay wells A2 to A11 (rows 7 to 16) are assigned the Vehicle control group type in the CSV Import file.









	1	2	3	4
A				
B				

Figure 29. A V denotes assay wells assigned to the Vehicle control group type.

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

- Type *Rot/AA* in column B for the appropriate rows/wells (**Figure 30**) to designate those wells as the Rotenone + Antimycin group in your assay template file (**Figure 31**).

1		<b>Assay Information</b>	
2	Project Name	Principal Investigator	Plated By
3	New XF Project Name		
4		<b>Assay Groups</b>	
5	Group Name	Control Group Type	Well
6	Background		A1
7	Rotenone + Antimycin Group Name	Rot/AA	A2
8	Rotenone + Antimycin Group Name	Rot/AA	A3
9	Rotenone + Antimycin Group Name	Rot/AA	A4
10	Rotenone + Antimycin Group Name	Rot/AA	A5
11	Rotenone + Antimycin Group Name	Rot/AA	A6
12	Rotenone + Antimycin Group Name	Rot/AA	A7
13	Rotenone + Antimycin Group Name	Rot/AA	A8
14	Rotenone + Antimycin Group Name	Rot/AA	A9
15	Rotenone + Antimycin Group Name	Rot/AA	A10
16	Rotenone + Antimycin Group Name	Rot/AA	A11
17	Background		A12
18			B1

Figure 30. Assay wells A2 to A11 (rows 7 to 16) are assigned the Rot/AA control group type in the CSV Import file.





	1	2	3	4
A				

Figure 31. An R denotes assay wells assigned to the Rotenone + Antimycin A control group type.

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

11 Add group names for all well positions (column A, row 6 through 101, [Figure 32](#)).

NOTE

A group name is required for all well positions, if a well is left blank (empty) you will see an error ([Figure 39](#)).

	A	B	C
1		<b>Assay Information</b>	
2	Project Name	Principal Investigator	Plated By
3	New XF Project Name		
4		<b>Assay Groups</b>	
5	Group Name	Control Group Type	Well
6	Background		A1
7	Group 2		A2
8	Group 3		A3
9	Group 4		A4
10	Group 5		A5
11	Group 6		A6
12	Group 7		A7
13	Group 8		A8
14	Group 9		A9
15	Group 10		A10
16	Group 11		A11
17	Background		A12
18	Group 1		B1
19	Group 2		B2
20	Group 3		B3
21	Group 4		B4
22	Group 5		B5
23	Group 6		B6
24	Group 7		B7
25	Group 8		B8
26	Group 9		B9

Figure 32. Example of group names assigned to each well (row).



## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

- 12 Save a copy of the Excel file as a comma delimited (CSV) file (Figure 33), then close the Excel CSV file before importing to Wave Pro or XF Pro.

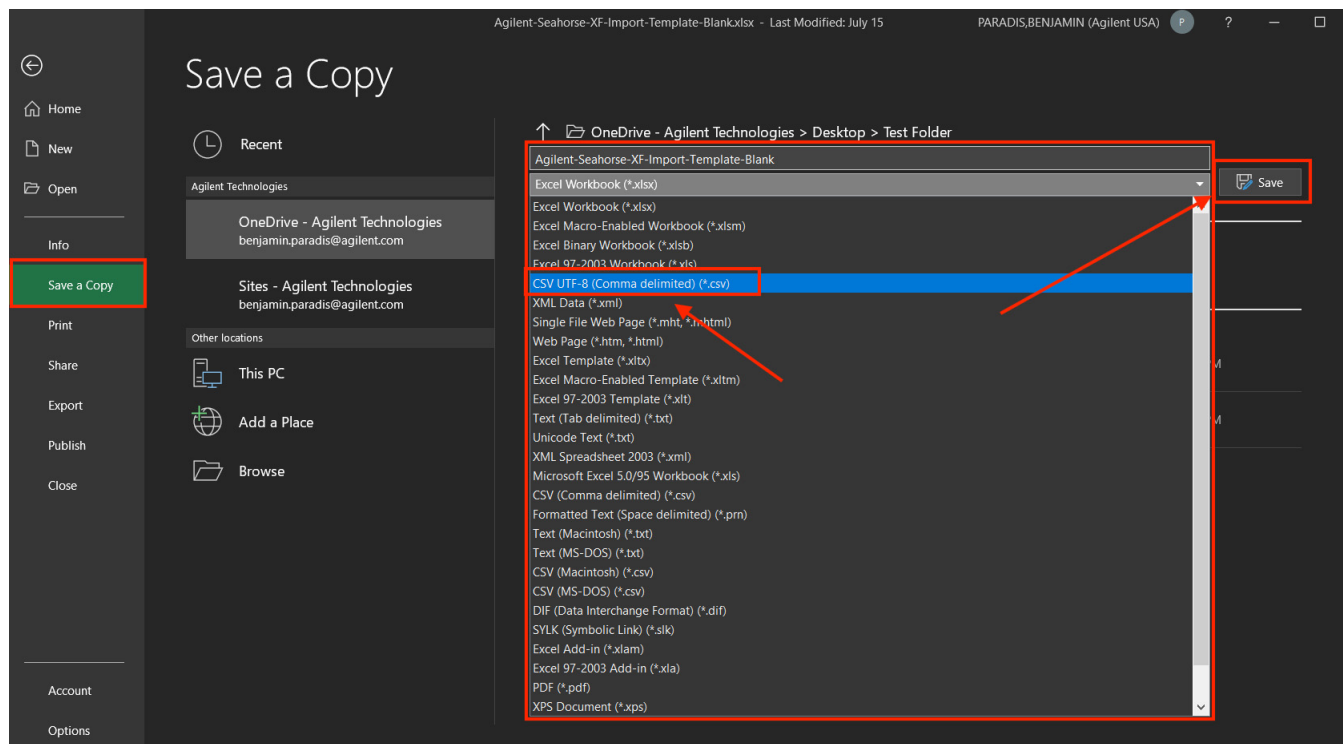


Figure 33. Save the Excel file as a CSV file.

- 13 Now in Wave Pro or XF Pro software, click **Import** (Figure 34), browse to the Excel CSV file, and click **Open** (Figure 35) to begin the template creation process (Figure 36). The time to complete the template creation process depends on the amount of information in the CSV file. In most cases, the process takes less than 1 minute.

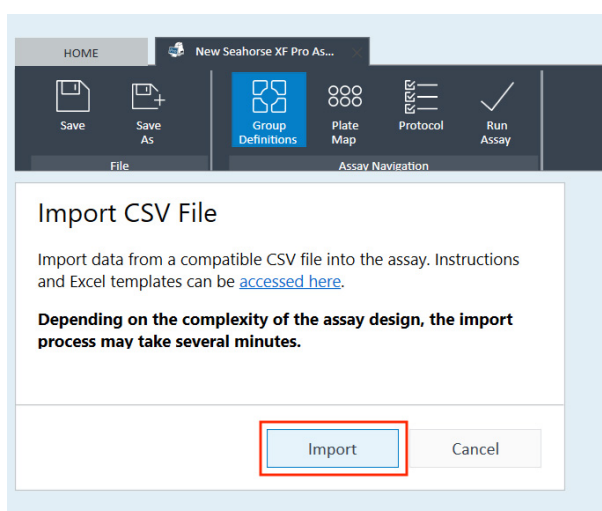


Figure 34. Import CSV view.

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

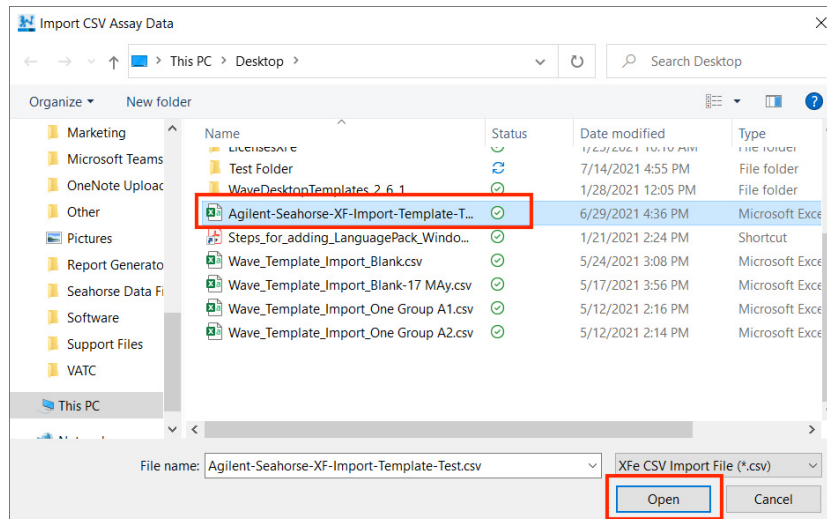


Figure 35. Locate the CSV file and click Open to begin the template creation process.

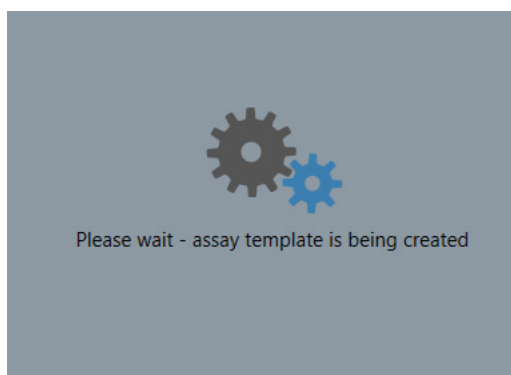


Figure 36. Wave Pro and XF Pro will display this message during the template creation process.

- 14 Once the template creation process is complete, you will automatically return to the Group Definitions view (not pictured), where you can review the list of groups created from the CSV file.

### NOTE

After the template creation process is finished, do not use the Generate Groups button found on the Group Definitions view. This function will overwrite the groups and plate map assignments automatically created using the CSV import feature.

## 2 Design XF Assays with Wave

Import group definitions and plate map layout using the CSV import feature

On the Plate Map view (**Figure 37**) you can review the list of groups AND group assignment on the plate map.

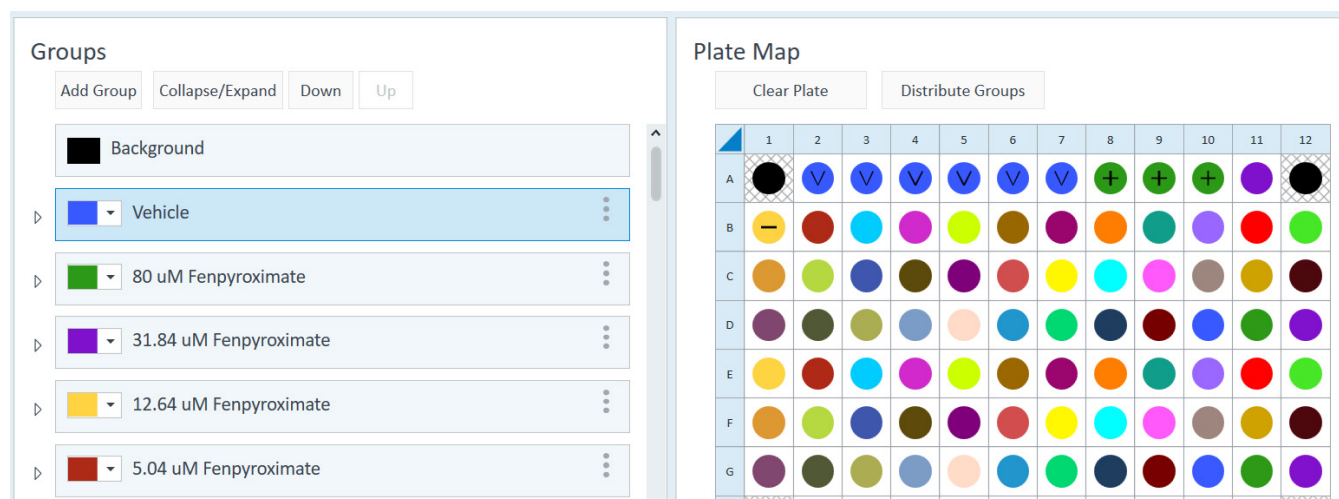


Figure 37. The plate map view in Wave Pro (or XF Pro) showing the list of groups (left) and the plate map assignment of those groups (right).

- 15 To finish your assay template, go to the Instrument Protocol view (**Figure 38**) and add the appropriate protocol steps to your template.



Figure 38. Instrument protocol view in Wave Pro and XF Pro software.

- 16 If you are using your PC & Wave Pro software to create your template, save the assay template file to a USB flash drive or shared network directory, then import to the XF Pro Analyzer. If you created the assay template using Wave Pro or XF Pro software (on the XF Pro controller), you can begin your assay when ready.

## 2 Design XF Assays with Wave

### Import group definitions and plate map layout using the CSV import feature

#### Other important information

The Excel CSV import file must contain the following minimum data to be a valid import file: (1) project name and (2) group names. If this information is not contained in the Excel CSV import file, Wave Pro and XF Pro will display an error message (**Figure 39**).



Figure 39. Error message displayed when project name and/or assay wells (rows) are not assigned a group. This message also appears if a language compatibility issue is detected.

Close your Excel CSV import file prior to Wave Pro or XF Pro import. If the Excel CSV file is left open, the import process cannot start and Wave Pro or XF Pro will display an error (**Figure 40**).

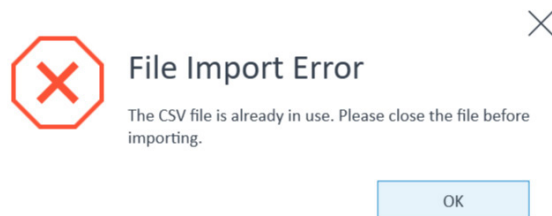


Figure 40. Error message displayed by Wave Pro and XF Pro if the Excel CSV import file is not closed prior to import.

The language locale of the PC used to create your Excel CSV import file must be the same language locale of the PC with Wave Pro software, where you will import the Excel CSV import file. If there is a language compatibility issue detected, you will see this error message (**Figure 39**)

- If a PC with a Windows OS language locale (e.g., French) is used create the Excel CSV import file, this Excel CSV import file can only be imported to Wave Pro software on a PC with the same French Windows OS language locale.
- If the same PC is used to create the Excel CSV import file, then to create the assay template with Wave Pro, you will not encounter any language compatibility issue.

Wave Pro software can be installed on the XF Pro Controller (computer) and any Windows PC. Wave Pro software is validated for compatibility with Windows OS languages for: English, German, French, Chinese (Traditional) and Japanese.

XF Pro Controller software can only be installed on the XF Pro Controller (computer). The XF Pro Controller Windows OS is set to the "English" language locale and must never be changed.

## Step 2: Plate Map

The Plate Map displays group assignments on the Cell Plate. The software uses the group assignments on the Plate Map to calculate group statistics (average rates for each measurement performed and the standard deviation or standard error for each rate measurement, etc.) after the assay has completed. There are two ways to assign groups to the Plate Map.

### Assign groups automatically

Click Distribute Groups. The software will automatically determine the maximum number of replicates per group, and assign wells to each group, beginning with column 1. (**Figure 41.**)

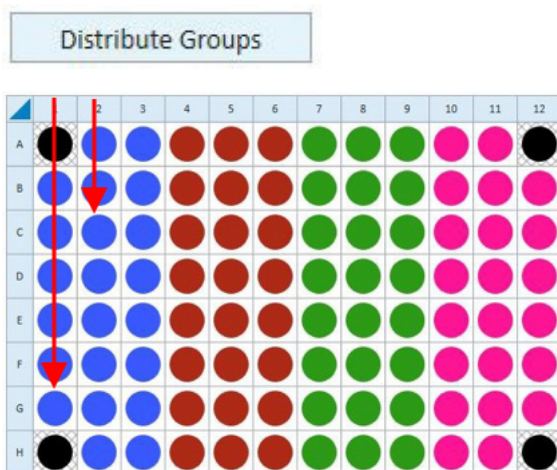


Figure 41. Distribute Groups

### Assign groups manually

- 1 Click the group in the Groups list on the left.
- 2 Click the well on the Plate Map to assign that individual well to the selected group. You can assign an entire row or column to a group by clicking the row or column label or drag-and-drop to select an area of the Plate Map to assign to the selected group. (**Figure 41.**)

### Background wells

Background wells have default Plate Map coordinates depending whether you are using an XF Pro Analyzer or an XF HS Mini Analyzer. You can modify the coordinates of the background wells or add/remove background wells if desired. Use the steps described in "**Assign groups manually**" on page 33.

## Step 3: Instrument Protocol

The Instrument Protocol is the series of commands performed during an assay, the timing of when these commands occur, and the duration. The default Seahorse assay templates have a preconfigured Instrument Protocol, and do not require modification.

### Default protocol commands

Every Instrument Protocol includes the following steps:

- Calibrate (always ON)
- Equilibrate (highly recommended for every assay)
- Baseline Measurement cycle

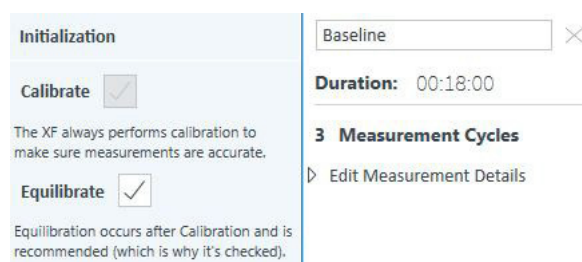


Figure 42. Default protocol commands

### Calibration

Calibrate is always the first step in a protocol and cannot be disabled. The Calibrate step reads the coefficients of the Sensor Cartridge and Cell Plate to ensure accurate data acquisition.

### Equilibration

Equilibration ensures temperature stability before beginning an assay. The default setting for Equilibration is **ON**. Equilibrate can be disabled although this is strongly discouraged.

### Measurement cycles

Measurement cycles are the steps in the Instrument Protocol when data is collected for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The standard Seahorse Instrument Protocol consists of three measurement cycles before the first injection (called Baseline) and three measurement cycles after each port injection. Each measurement cycle consists of three commands: Mix, Wait, and Measure.

- **Mix:** The amount of time to raise/lower the sensor cartridge to ensure analytes and drug compounds are uniformly mixed in each well before/after an injection, as well as to reintroduce oxygen to the well after forming the microchamber for each measurement.
- **Wait:** The amount of time to delay the Measurement step after the Mix step. This command is not required for the XF Pro Analyzer and the XF HS Mini Analyzer, but may be added to Instrument Protocol if necessary.
- **Measure:** The amount of time to record the flux of analytes in the transient microchamber once the sensor cartridge probes are lowered following a Mix (or Wait) command.

The default measurement cycle times for the XF Pro Analyzer and XF HS Mini Analyzer are:

## 2 Design XF Assays with Wave Injections

- Mix: 3 minutes
- Wait: 0 minutes
- Measure: 3 minutes

### Baseline measurement cycle

The Baseline measurement cycle is the starting point for every Seahorse assay and consists of three measurements before the first injection. Data acquired during the Baseline measurement cycle provide valuable information about the bioenergetic status of the cells under your starting conditions.

Baseline

Duration: 00:18:00

**3 Measurement Cycles**

▲ Edit Measurement Details

Cycles	Mix	Wait	Measure
▲	▲	▲	▲
3	03:00	00:00	03:00
▼	▼	▼	▼

Figure 43. Default cycles and timing

## Injections

To add an injection command to the Instrument Protocol, click **Injection**. The software automatically selects the compound injection ports sequentially. Therefore, the first time you click **Injection**, port A will be assigned to injection 1. A grayed-out port indicates it has already been assigned to an injection. (Figure 44.)

Oligomycin

Duration: 00:18:00

**3 Measurement Cycles**

Select Ports

A B

C D

Measure After Injection ☒

▲ Edit Measurement Details

Cycles	Mix	Wait	Measure
▲	▲	▲	▲
3	03:00	00:00	03:00
▼	▼	▼	▼

Figure 44. Measurement information

The software also automatically adds the measurement cycles after adding an injection. Default **Mix**, **Wait**, and **Measure** times are instrument-dependent, and can be modified in **Instrument Options** (Wave Home > Options).

## Edit measurement details

To view and edit the **Mix**, **Wait**, and **Measure** timing, click the drop-down arrow next to **Edit Measurement Details**. (Figure 44.) Use your keyboard to enter specific **Mix**, **Wait**, and **Measure** times or adjust the number of **Cycles** for each measurement. The up and down arrows can also be used. To achieve optimal results, a minimum of three cycles per measurement is recommended. The default **Mix**, **Wait**, and **Measure** times are instrument-dependent, and can be modified in Instrument Options (**Wave Home > Options**).

To change the name of each command in the **Instrument Protocol**, click the text field at the top of the column. (Figure 44.)

## Custom cycles

Most Seahorse assays do not require the use of custom cycles, however should your assay require a custom cycle this must be added to your Instrument Protocol. To add a custom cycle command to the **Instrument Protocol**, click **Custom**. A custom cycle enables multiple Mix and Wait steps without a Measurement. Note that the Agilent Seahorse XF Report Generators are unable to analyze result data that contains a custom cycle.



## Step 4: Run Assay

### Assay summary

#### General Information

The General Information section provides editable fields for project information, plate information, or other important notes related to the assay. Any information entered on this view is saved in the assay result file (.xflr).

#### Assay Summary

Project Information	Plate Information	Notes
<p>Project Name</p> <input type="text"/>	<p>Plated By</p> <input type="text"/>	<div></div>
<p>Principal Investigator</p> <input type="text"/>	<p>Plated On</p> <div>Select Date <input type="text" value="15"/></div>	
<p>Project Number</p> <input type="text"/>		
<input type="checkbox"/> Show Advanced Options		

Figure 45. Assay summary

### Errors and Warnings

All errors or warnings are displayed on the right side of the Run Assay step. (See [Figure 46](#).) Prior to transferring the template to the XF Pro or XF HS Mini Analyzer, correct any errors that are displayed. A typical error message is the notification that some assay wells have not been assigned to a group on the Plate Map. If a template is designed correctly, the software displays an All Set confirmation message. (See [Figure 46](#).)




<div></div> <div><b>All Set</b> Your experiment can now be run. Transfer the assay template file to your analyzer using a USB flash drive or shared network directory.</div>	<div></div> <div><b>Warning</b> We cannot run this experiment <b>At least one group must be defined</b> <b>At least one well must be assigned to a group</b></div>	<div></div> <div><b>Alert</b> <b>No background wells are defined</b> <b>Some wells are not assigned to a group</b> <b>No injection ports were included in the assay protocol.</b></div>
---	---	--

Figure 46. Errors & warnings

## Advanced options

### Email settings (XF Pro Controller software only)

This feature applies to XF Pro Controller software only and requires an active internet connection on the XF Pro Controller. Recipients must be added before starting an assay. Wave Pro software does not email assay result files.

To add an email address:

- 1 Open the template file.
- 2 On the **Run Assay** navigation step, click **Show Advanced Options**. (Figure 47.)

☒ Show Advanced Options

Email Settings		Version Information	
Email address	Add	Software	10.0.1.33
		File	8

Figure 47. Advanced Options

- 3 Type in the email address below **Email Settings**, and click **Add**.
- 4 Repeat for each email address.

### Version information

**Version Information** displays the software version and **File** version info (internal-use only). (See Figure 47.)

### Protocol summary

To display a summary view of the Instrument Protocol, click **Protocol**.

## 2 Design XF Assays with Wave

### Advanced options

(See [Figure 48](#).) To edit the Instrument Protocol, click the Instrument Protocol button in the Assay Navigation ribbon before running the assay.

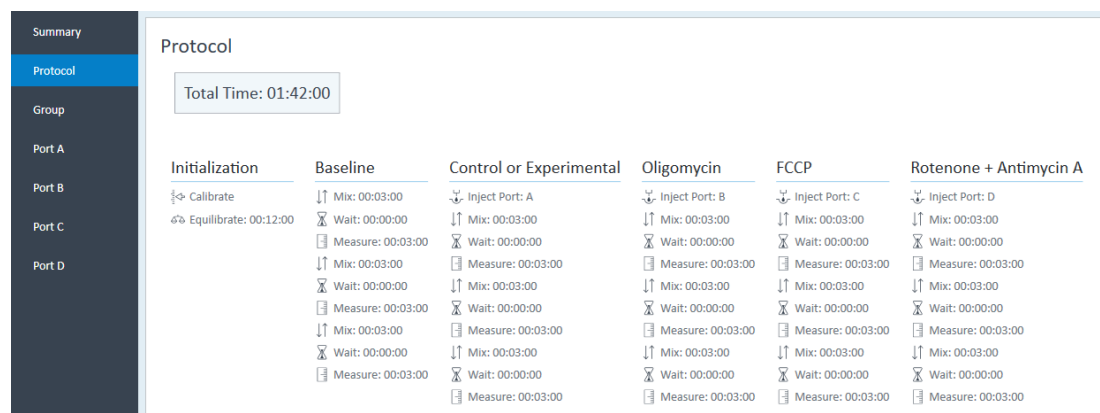


Figure 48. Instrument protocol summary

### Group summary

To display a summary view of the Group Definitions and Plate Map, click **Group**. (See [Figure 49](#).) To edit the Group Definitions or Plate Map, click the **Group Definitions** or **Plate Map** navigation step before running the assay.

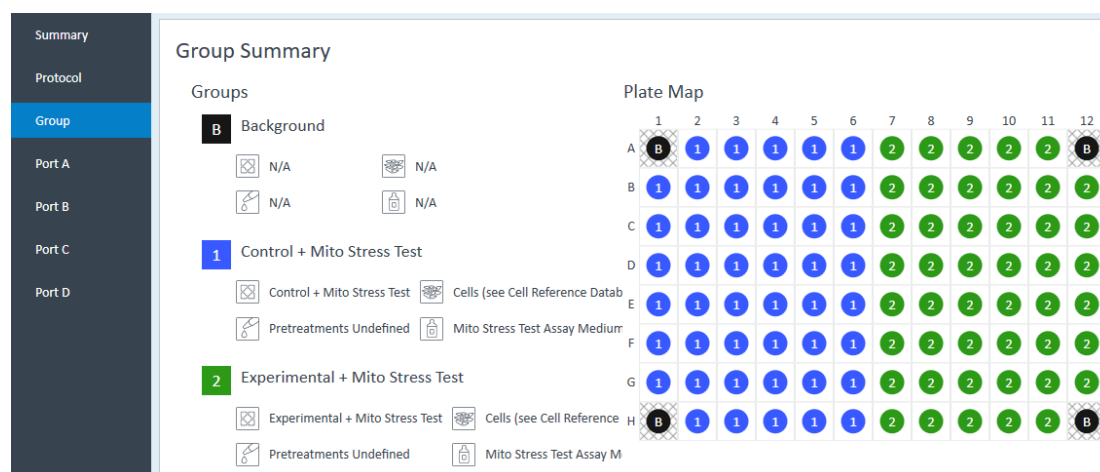


Figure 49. Group definitions & plate map summary

### Port (A, B, C, or D) summary

Click Port **A** (**B**, **C**, or **D**) for a summary of each injection from the selected port. (See [Figure 50](#).) The summary information includes:

- Compound concentration
- Name of the solvent (if specified)
- Percentage of solvent used for each compound
- Port volume

## 2 Design XF Assays with Wave

### Advanced options

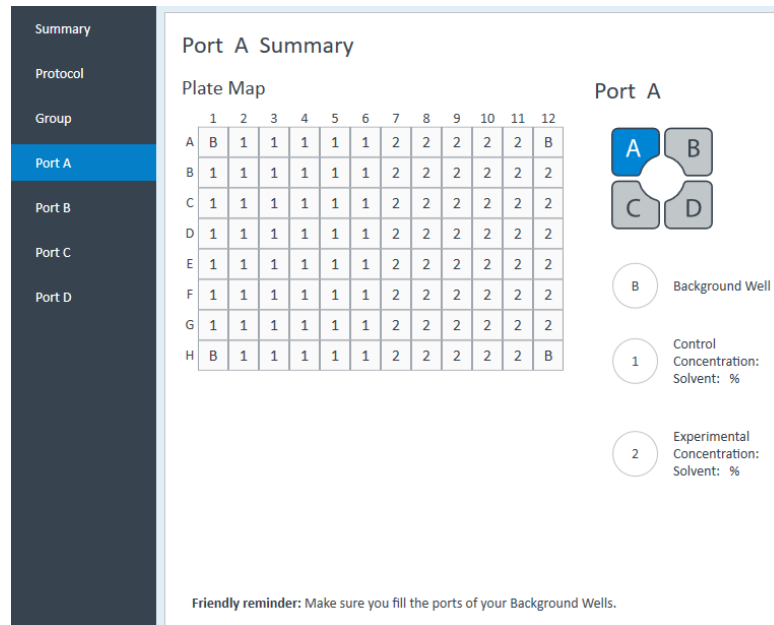


Figure 50. Injection summary for Port A

To modify the compound name, compound concentration, solvent name or percentage, click the Group Definitions navigation step, then select the appropriate Injection Strategy to edit before running the assay.

## Use titration setup to create groups and plate map layout

Titration setup is a template design feature available in both Wave Pro software and XF Pro Controller software. If you intend to graph result data as a dose response curve in Wave Pro & Seahorse Analytics software, assay groups MUST be created using the titration setup feature.

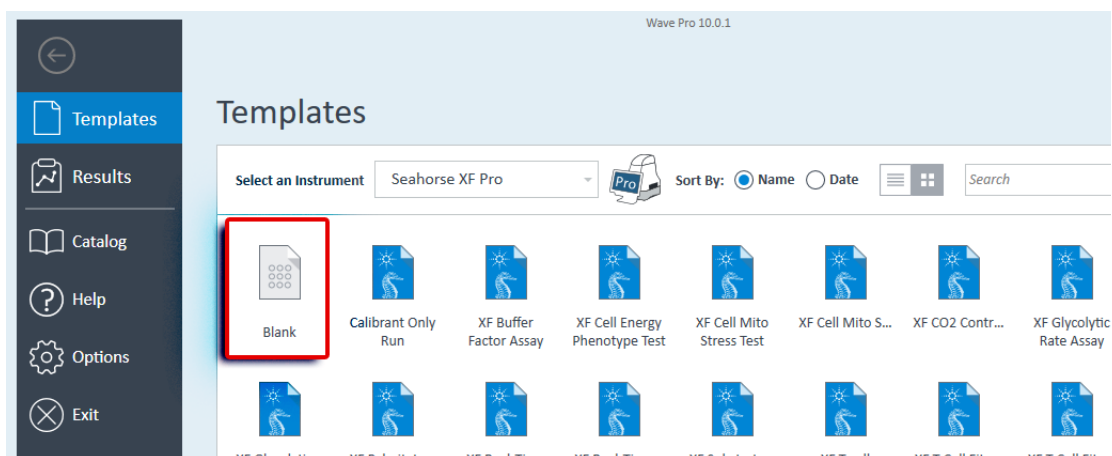
Titration setup allows you to generate a collection of groups under a common titrate or compound name, with a set dilution strategy and color gradient from highest to lowest compound concentration.

A dose (or concentration) will be automatically calculated and assigned to each group in the titrate series based on the user-defined starting concentration, dilution factor and other information.

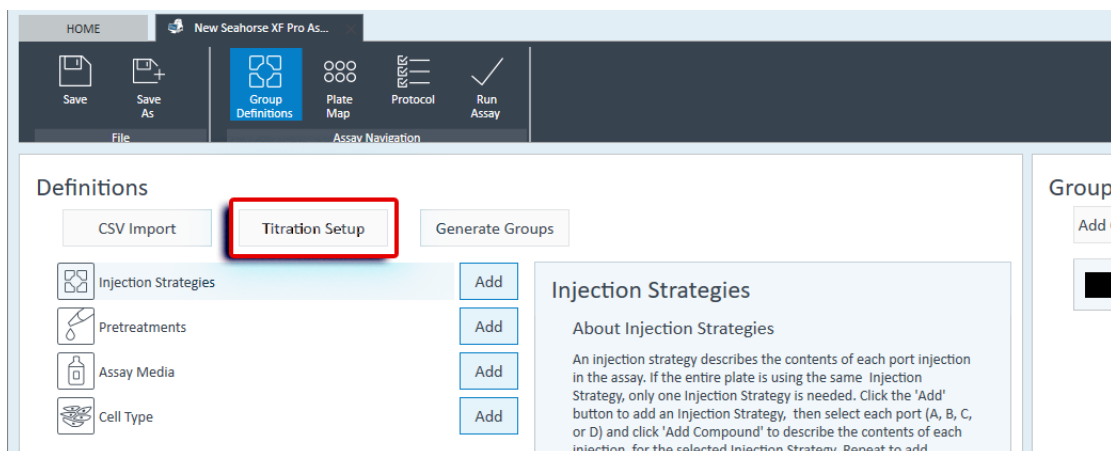
### Create titration series

#### Create a new assay template

- 1 Open the **Blank** template



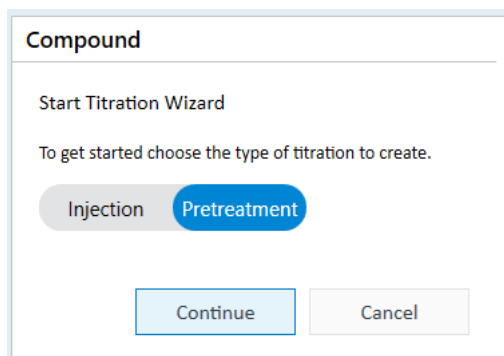
- 2 Select **Titration Setup**



## 2 Design XF Assays with Wave

### Create titration series

- 3 Choose the type of titration- **Injection** or **Pretreatment**- and click **Continue**. In this example, we will select **Pretreatment**.



**Compound**

Start Titration Wizard

To get started choose the type of titration to create.

Injection Pretreatment

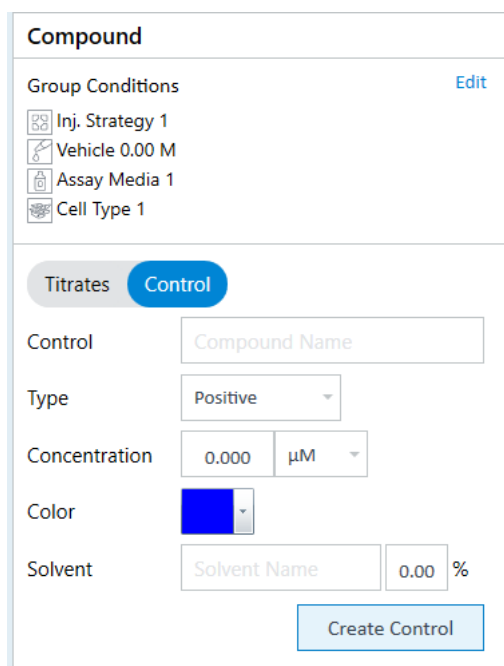
Continue Cancel

### NOTE

Before creating your titration, you must add group definitions. Since the Titration Setup will automatically generate groups, adding the definitions now ensures all groups automatically generated contain this important assay information. See [“Step 1: Group Definitions”](#) on page 16.

### Create Vehicle and Rot/AA groups

- 1 Type “Vehicle” in the **Control** field



**Compound**

Group Conditions [Edit](#)

- Inj. Strategy 1
- Vehicle 0.00 M
- Assay Media 1
- Cell Type 1

Titrates Control

Control Compound Name

Type Positive

Concentration 0.000 µM

Color Blue

Solvent Solvent Name 0.00 %

Create Control

## 2 Design XF Assays with Wave

### Create titration series

- 2 Select **Vehicle** from the **Type** drop down

**Compound**

Group Conditions [Edit](#)

- Inj. Strategy 1
- Vehicle 0.00 M
- Assay Media 1
- Cell Type 1

**Controls**

**Titrates** **Control**

Control: Vehicle

Type: Vehicle

Concentration: 0.000 μM

Color: Blue

Solvent: Solvent Name 0.00 %

[Create Control](#)

- 3 You can select a color for this control group from the **Color** drop-down.
- 4 Click **Create Control** to add to the group to the titration list.
- 5 Repeat these steps to create the Rot/AA titration, entering Rot/AA in the **Control** field and selecting Rot/AA as the **Type** for this group.

## Assign groups to the plate map

- 1 Select a titration group from the list

**Compound**

Group Conditions [Edit](#)

- Inj. Strategy 1
- Vehicle 0.00 M
- Assay Media 1
- Cell Type 1

**Titration**

- Background
- Vehicle
- Rot/AA

**Platemap**

Wells Used: 4  
Wells Remaining: 92

- 2 Click on the plate map wells to assign each group as shown in [Figure 51](#).

**Titration**

- Background
- Vehicle
- Rot/AA

**Platemap**

Autofill Options: ☒ Decreasing ☐ Increasing ☒ Horizontal ☐ Vertical

Wells Used: 16  
Wells Remaining: 80

[Clear Plate](#)

[Done](#) [Delete All](#)

Figure 51. Vehicle and Rot/AA group plate assignment

### NOTE

Failure to assign the Vehicle and Rot/AA groups as shown in [Figure 51](#) will disable access to the XF Mito Tox data analysis tools.



## 2 Design XF Assays with Wave

### Create titration series

#### Create compound titration series groups

- 1 Select Titrates.

**Compound**

Group Conditions [Edit](#)

☐ Inj. Strategy 1  
☐ Vehicle 0.00 M  
☐ Assay Media 1  
☐ Cell Type 1

**Titrates** Control

Compound

Dilution **Serial** Step

Dilution Factor  fold # Doses

Max Concentration   $\mu\text{M}$

Color

Solvent   %

[Create Titration](#)

- 2 Enter a compound name for Compound 1 in the **Compound** field.

**Compound**

Group Conditions [Edit](#)

☐ Inj. Strategy 1  
☐ Vehicle 0.00 M  
☐ Assay Media 1  
☐ Cell Type 1

**Titrates** Control

Compound

Dilution **Serial** Step

Dilution Factor  fold # Doses

Max Concentration   $\mu\text{M}$

Color

Solvent   %

[Create Titration](#)

- 3 Select **Series** or **Step** dilution
- 4 Enter the **Dilution Factor**
- 5 Enter the number of **Doses**

## 2 Design XF Assays with Wave

### Create titration series

- 6 Enter the **Maximum Compound Concentration** and the appropriate measurement units from the drop-down

Compound

Group Conditions [Edit](#)

Inj. Strategy 1

Vehicle 0.00 M

Assay Media 1

Cell Type 1

Titrates

Control

Compound

Compound 1

Dilution

Serial

Step

Dilution Factor

2.000

fold

# Doses

10

Max Concentration

100.000

mM

Color

Solvent

Solvent Name

0.00

%

Create Titration

- 7 Select a color from the Titration group as desired

- 8 Click **Create Titration**

The software automatically generates a collection of groups. This Compound list includes the individual titrations for the compound

Titrations		
	Background	
▷	Vehicle	
▷	Rot/AA	
◀	Compound 1	
1	Compound 1 0.100 M	0.100 M
2	Compound 1 0.050 M	0.050 M
3	Compound 1 0.025 M	0.025 M
4	Compound 1 0.013 M	0.013 M
5	Compound 1 0.006 M	0.006 M
6	Compound 1 0.003 M	0.003 M
7	Compound 1 0.002 M	0.002 M
8	Compound 1 0.001 M	0.001 M
9	Compound 1 3.91E-4 M	3.91E-4 M
10	Compound 1 1.95E-4 M	1.95E-4 M

#### NOTE

Do not modify the default group names generated by the titration setup. This will break compatibility with the dose response graphs in Wave Pro and Seahorse Analytics and data will not be presented in a dose response curve.

## Assign compound lists to the plate map using Auto Fill

The **Autofill Options** are located at the top of the Platemap. Autofill options determine the placement (horizontal or vertical) and sorting (concentration from lowest to highest or vice versa) of the individual titrations on the plate map when assigning compound groups (**Figure 52**).

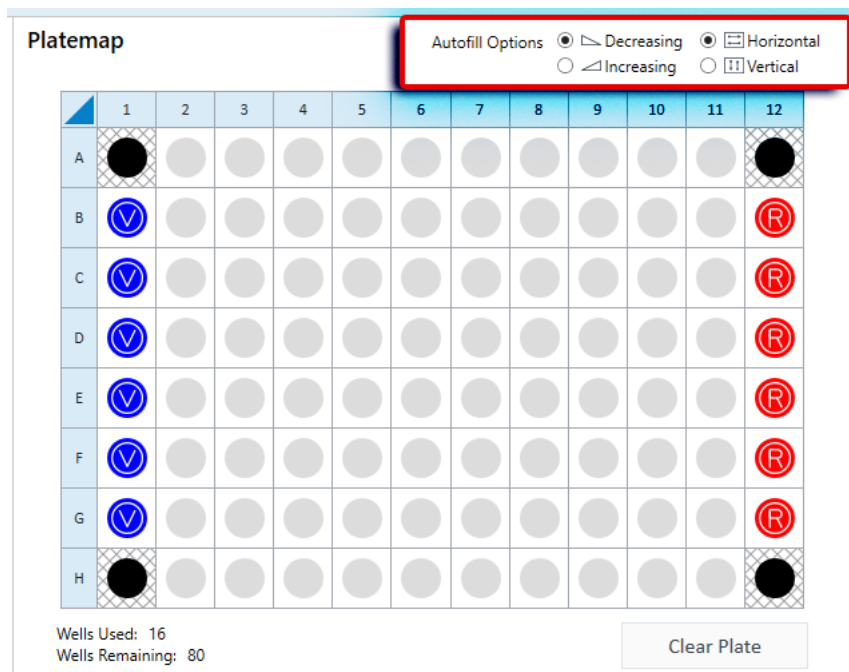
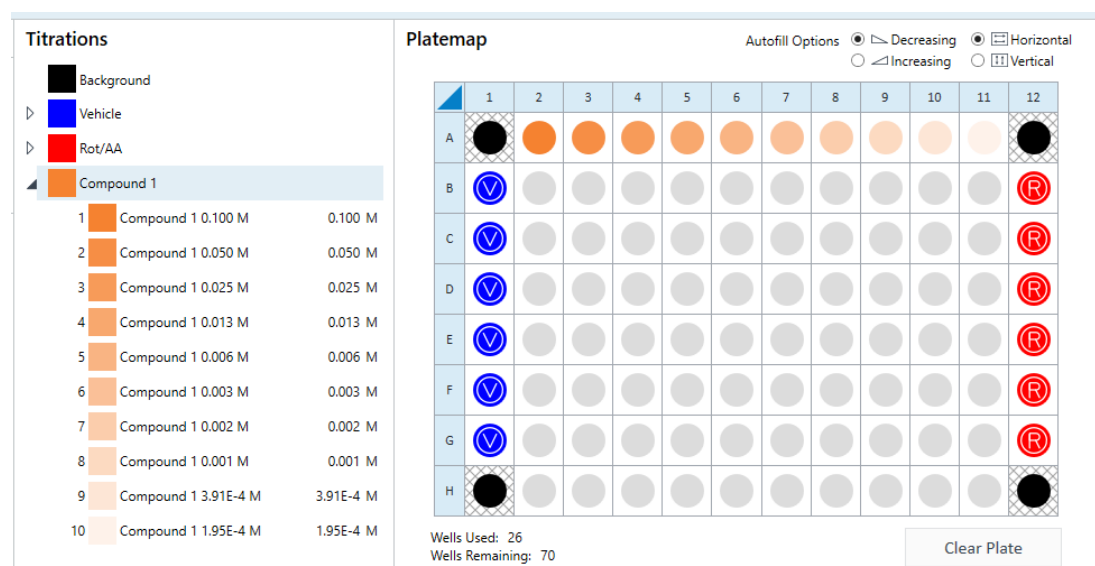


Figure 52. Autofill Options

- 1 Select a compound group from the **Titration** list.
- 2 Select **Autofill Options** as desired
- 3 Click in the first well location of the plate map to add the compound group as configured in the Autofill Options. In this example, location A2 is selected.



## 2 Design XF Assays with Wave

### Create titration series

Continue to create remaining Compound Lists and add them to the Plate map.

The screenshot displays the Wave Pro software interface with two main panels: **Titration** and **Platemap**.

**Titration Panel:** A list of titration series is shown on the left, each with a colored square icon. The series include: Background (black), Vehicle (blue), Rot/AA (red), Compound 1 (orange), Compound 2 (green), Compound 3 (teal), Compound 4 (purple), Compound 5 (brown), Compound 6 (dark teal), Compound 7 (red-orange), and Compound 8 (yellow). The 'Compound 8' series is currently selected.

**Platemap Panel:** A 12x8 grid (columns 1-12, rows A-H) represents the plate layout. The grid is populated with colored circles corresponding to the titration series. The corners (A1, A12, H1, H12) are black, representing background wells. The first column (A1-H1) contains blue circles with a 'V' (Vehicle). The last column (A12-H12) contains red circles with an 'R' (Rot/AA). The remaining wells are filled with colored circles representing the various compounds. Above the grid, 'Autofill Options' are set to 'Decreasing' and 'Horizontal'. Below the grid, it indicates 'Wells Used: 96' and 'Wells Remaining: 0'. A 'Clear Plate' button is located at the bottom right of the grid.

At the bottom of the interface, there are two buttons: 'Done' and 'Delete All'.

4 Click **Done** when you are finished.

You can review and edit the list of **Groups** created. Groups are listed under **Group Definitions**.

The screenshot displays the Wave Pro software interface with the **Group Definitions** and **Groups** panels.

**Group Definitions Panel:** This panel is divided into three tabs: 'CSV Import', 'Titration Setup', and 'Generate Groups'. The 'Generate Groups' tab is active. It shows a list of groups on the left, including 'Injection Strategies' (Inj. Strategy 1) and 'Pretreatments' (Vehicle 0.00 M, Rot/AA 0.00 M, Compound 1 0.100 M, Compound 1 0.050 M, Compound 1 0.025 M, Compound 1 0.013 M, Compound 1 0.006 M, Compound 1 0.003 M, Compound 1 0.002 M, Compound 1 0.001 M, and Compound 1 3.91E-4 M). The 'Compound 1 0.100 M' group is selected. To the right of the list is an 'Edit Pretreatment' form with fields for Name, Description, Compound Catalog, Compound, Final Concentration (100.000 mM), and Solvent (0.00 %).

**Groups Panel:** This panel shows a list of groups on the right, including 'Background', 'Vehicle 0.00 M', 'Rot/AA 0.00 M', 'Compound 1 0.100 M', and 'Compound 1 0.050 M'. Each group has a dropdown menu for 'Inj. Strategy 1' and 'Assay Media 1', and a dropdown menu for 'Cell Type 1'. The 'Compound 1 0.100 M' group is selected.

After you have created your titration series and assigned them in the plate map, you can save your assay template, see **"Saving Assay Templates"** on page 49.

## Saving Assay Templates

To save your assay template, click the **Save** or **Save As** button. If you are editing a Seahorse assay template, these template details cannot be overwritten, therefore you will be prompted to save a new template file. If you are editing a user-generated template file, clicking Save will overwrite the template with the new details/content. To preserve the original user-generated assay template, click the **Save As** button to create a new template file.



Figure 53. Save and Save as options

Click **Save As** to display the **Save As Template** window (See [Figure 54](#)):

- 1 Enter the name of the assay template in the Name field (required).
- 2 Enter the Author name (optional).
- 3 Enter a description of the assay in the Description field (optional).
- 4 Click **Save**.

### Save As Template

Name	<input type="text" value="Ben's Custom Template"/>
Author	<input type="text" value="BP"/>
Description	<input type="text"/>
<div><input type="button" value="Save"/> <input type="button" value="Cancel"/></div>	

Figure 54. Save As window

## Run Assay on the XF Pro Analyzer

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This chapter applies to XF Pro Controller software for the Seahorse XF Pro Analyzer ONLY.

### Creating an assay template file

Assay template files (.asyt) can be created using Wave Pro software, and using XF Pro Controller software. Assay templates created using XF Pro Controller software can be run immediately after finished creating the template on the XF Pro Analyzer. Assay templates created using Wave Pro must be imported to XF Pro Controller before you can start an assay.

## Transfer Assay Template(s) to Seahorse XF Pro Analyzer

### Transfer assay template using a USB flash drive

#### Option #1:

- 1 Save template to a USB flash drive.
- 2 Plug the flash drive into the USB port on the Seahorse XF Pro Controller.
- 3 Select the USB flash drive and locate the template file(s).
- 4 Double-click the template to import automatically to XF Pro Controller.

#### Option #2:

- 1 Save template to a USB flash drive.
- 2 Plug the flash drive into the USB port on the Seahorse XF Pro Controller.
- 3 Using XF Pro Controller software, click Import on the Templates view.
- 4 Select the USB flash drive and locate the template file.
- 5 Select one or multiple template files on the USB flash drive, then click Open.

### Transfer assay template using a shared network drive

#### Option #1:

- 1 Ensure that the XF Pro Analyzer Controller is ON.
- 2 Start XF Pro Controller software.
- 3 Locate the assay template file in the shared network directory.
- 4 Double-click the template to import automatically to XF Pro Controller.

#### Option #2:

- 1 Ensure that the XF Pro Analyzer Controller is ON.
- 2 Using XF Pro Controller software, click Import on the Templates view.
- 3 Locate the template file in the shared network directory.
- 4 Select one or multiple template files on the network drive, then click Open.

## Start Your Seahorse XF Pro Assay

When ready to begin your XF Pro assay, click Start Run in XF Pro Controller software.

### CAUTION

**Once an assay is started, access to other software applications or Seahorse files is disabled until the assay is complete.**

- 1 Click Start Run and select a location to save the assay template file (if it's a new template).

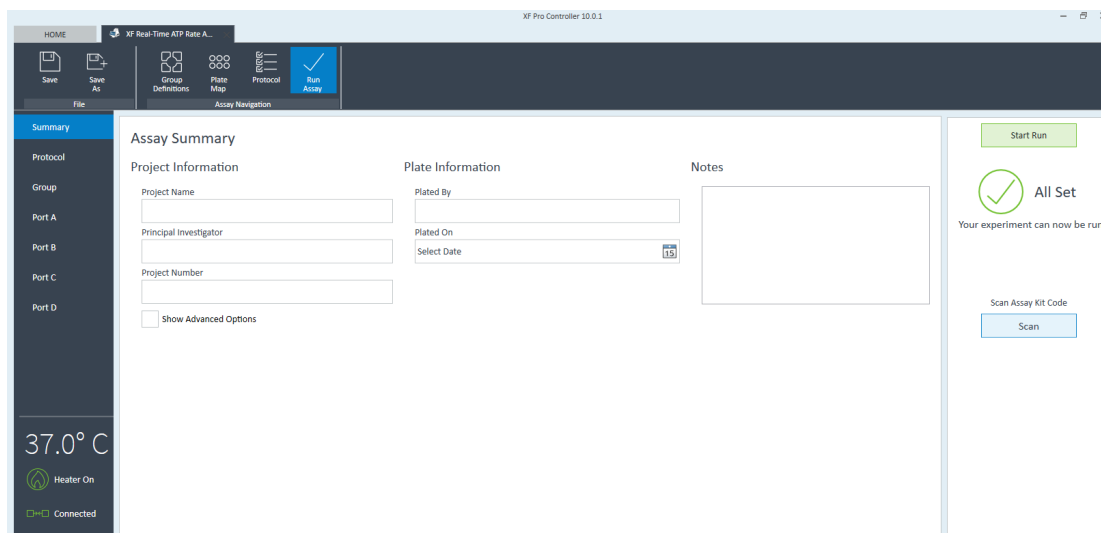


Figure 55. Click Start Run

The assay result file will be saved in the same location following assay completion (see [Figure 56](#)). Assay template and result files can be saved to a USB Flash Drive, shared network directory, or locally on the XF Pro Controller. The software will automatically save a backup



### 3 Run Assay on the XF Pro Analyzer

#### Start Your Seahorse XF Pro Assay

assay result file locally on the XF Pro Controller in the location: C:\ProgramData\Agilent Technologies, Inc\Seahorse Wave Controller\Assays.

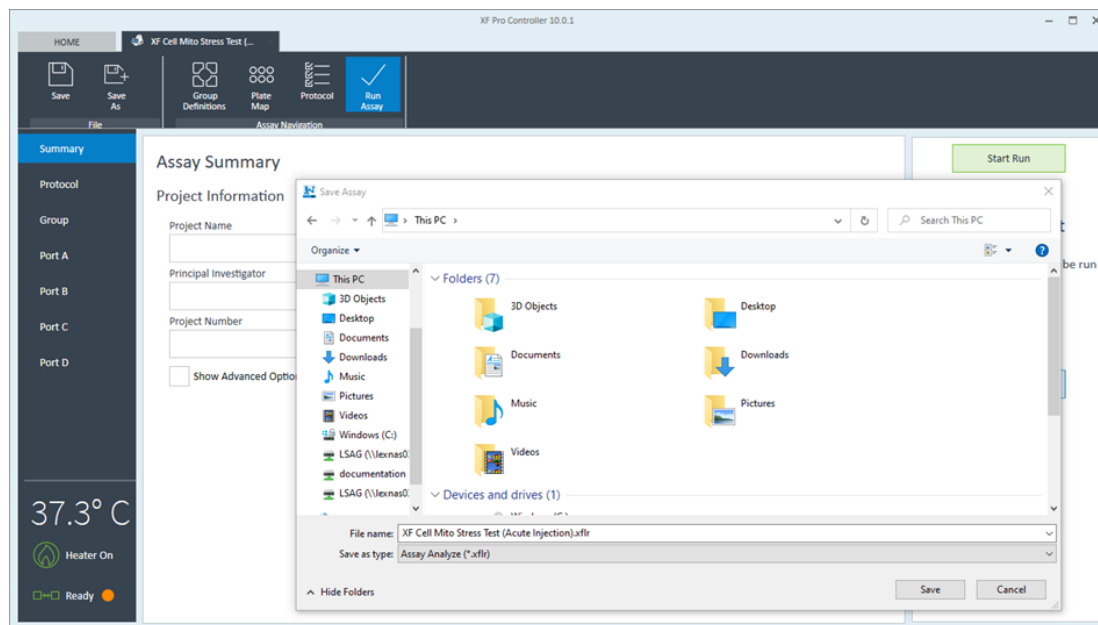


Figure 56. Save Assay

- 2 After selecting a save location, XF Pro Controller software will ask if you are ready to load the sensor cartridge and utility plate (**Figure 57**).

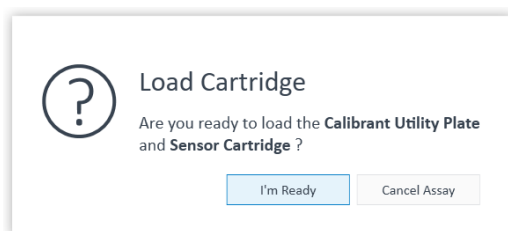


Figure 57. Load Cartridge prompt

Press **I'm Ready** to open the tray door and eject the thermal tray. Place the Sensor Cartridge (hydrated and loaded with compounds) along with the Utility Plate onto the tray.

#### NOTE

Prior to loading the XF Sensor Cartridge & Utility Plate, ensure the XF Hydrobooster is removed (**Figure 58**). For more information on this procedure, please see the official **XF Hydrobooster User Guide**.

### 3 Run Assay on the XF Pro Analyzer

#### Start Your Seahorse XF Pro Assay

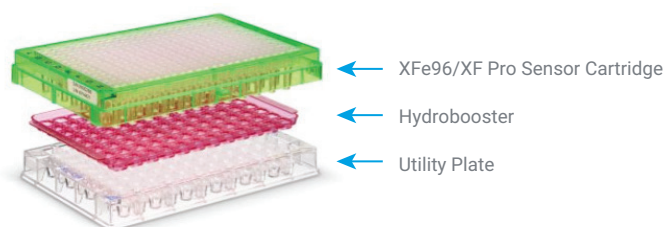


Figure 58. Assembly of the Agilent Seahorse XF Pro Hydrobooster with the Agilent Seahorse XFe96/XF Pro sensor cartridge and utility plate.

#### NOTE

**Ensure the Cartridge fits properly on the Utility Plate, the lid is removed from the Cartridge, and the direction of the Cartridge is in the proper orientation.**

- 3 After placing the Sensor Cartridge and Utility Plate on the thermal tray, press **Load Cartridge** to initiate Sensor Cartridge Calibration. The time to complete Calibration for assays at 37 °C is approximately 20-25 minutes. For assays performed at temperatures other than 37 °C, an additional 30 minutes of pre-calibration time will be added to ensure accurate data acquisition.

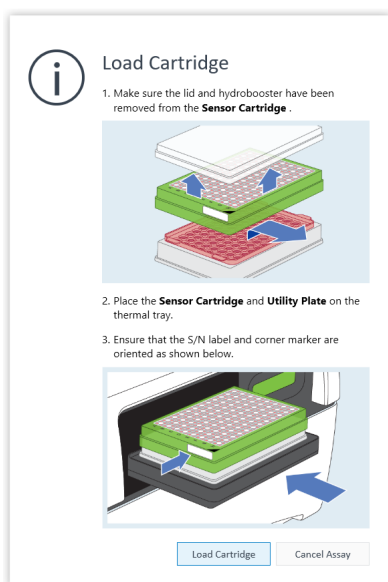


Figure 59. Load Sensor Cartridge + Utility Plate prompt

### 3 Run Assay on the XF Pro Analyzer

#### Start Your Seahorse XF Pro Assay

- 4 After completing Calibration, XF Pro Controller software will display the Load Cell Plate message prompt. Click Open Tray to eject the Utility Plate and load the Cell Plate. Ensure the lid is removed from the Cell Plate before loading.

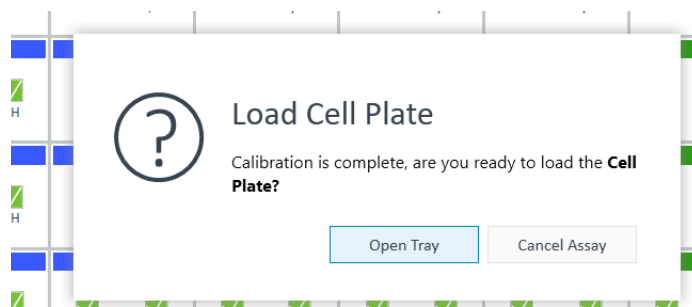


Figure 60. Load Cell Plate prompt

- 5 Click Load Cell Plate to initiate Equilibration. Once Equilibration has finished, the assay will begin acquiring the first Baseline measurement.

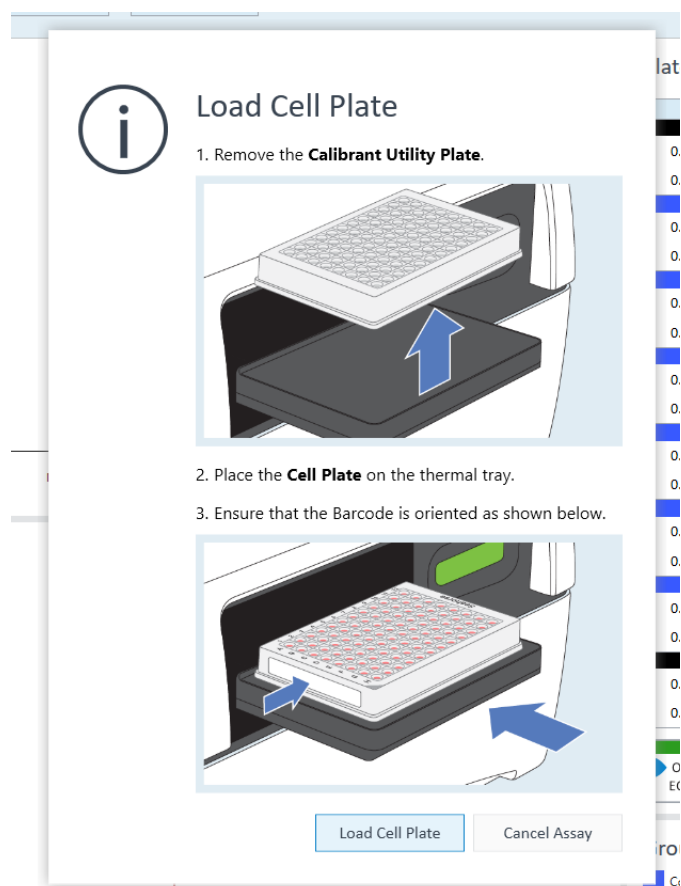


Figure 61. Load Cell Plate prompt

3 Run Assay on the XF Pro Analyzer  
Start Your Seahorse XF Pro Assay

- 6 After completing the final Instrument Protocol step, the assay will finish and display an Unload Sensor Cartridge message prompt. To eject the Sensor Cartridge and Cell Plate from the XF Pro Analyzer, press **Eject**.

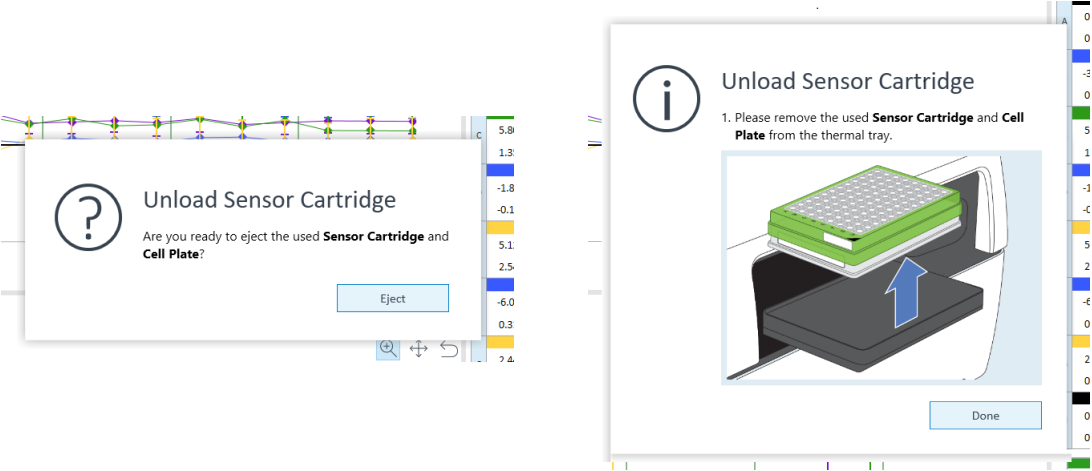


Figure 62. Unload Sensor Cartridge prompt

- 7 Once you have removed the used cell plate & sensor cartridge, you will be prompted to either view the result data now, or you can return to the Home view. Selecting the option to view results now will automatically open the result file in Wave Pro software on the XF Pro Controller. You can also transfer the result file to your personal computer for analysis with Wave Pro software or Seahorse Analytics (recommended).

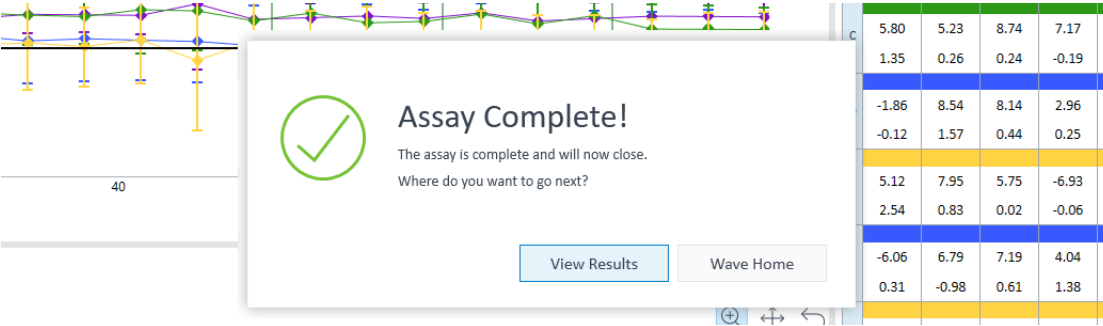


Figure 63. Assay Complete prompt

## Add and Remove Measurement Cycles during an assay

Figure 64. Data acquisition runtime display in XF Pro Controller software

During an XF Pro assay, rate data is acquired, calculated and presented in real-time. For certain assays, it may be necessary to add additional measurements, or remove measurement cycles based on real-time responses in prior measurements.

XF Pro Controller software enables real-time Instrument Protocol modification to steps in the protocol that have not yet been executed. To display the Instrument Protocol for the selected command, click on an **Instrument Protocol** step above the data. (See [Figure 65](#).)

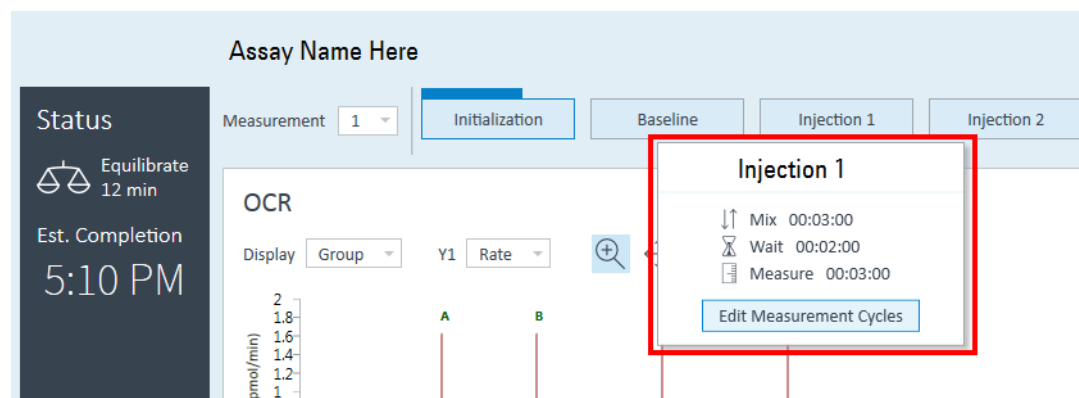


Figure 65. Display instrument protocol information

Click **Edit Measurement Cycles** to display the **Edit Number of Cycles** window. (See [Figure 66](#).) Use the up/down arrows (or manually type a number) to add/remove measurement cycles from the selected command.

### 3 Run Assay on the XF Pro Analyzer

Add and Remove Measurement Cycles during an assay

**Edit Number of Cycles**

Command: Injection 1  
Total Protocol Time: 00:56:00

Current step:  
This window will close in 120 Seconds

**Adjust Measurement Cycles**  
Increase or decrease the number of measurement cycles for the selected protocol step. Adding measurement cycles will increase length of the assay.

▲  
1  
▼

New Protocol Time:  
00:56:00

Save Cancel

Figure 66. Add and remove measurement cycles for a specific instrument protocol step

## XF Pro Controller Best Practices

- **Reboot the XF Pro Controller (computer) at least once per week.**  
You may notice at times the Windows OS or XF Pro Controller software responding slowly. This can be due to an application over-using the computer's resources (CPU, physical memory), memory leaks, or other low-level errors that have accumulated over time. Rebooting the XF Pro Controller (computer) resets the current state of Windows and software applications that may be causing any of the issues mentioned above.
- **Save & close open assay template files and assay result files prior to starting an XF assay.**  
Like any software program, more computer resources (CPU & memory) are consumed when multiple files are open in Wave Pro or XF Pro Controller software, even if the files are not actively being used. This could lead to instability of the software, or even the Windows OS itself.
- **Do not insert or eject USB flash drives (or insert/remove network cable) during an XF assay.**  
If the target file save location for the assay result file is the USB flash drive or virus protection software starts scanning the USB. This could lead to instability of XF Pro Controller software and in some instances may result in data loss.
- **Limit data analysis performed on the XF Pro Controller (computer).**  
XF Pro Analyzers acquire a significant amount of data for each assay, as mentioned above leaving files open in Wave Pro / XF Pro Controller software consumes computer resources, even if the files are not actively being used. Additionally, modifying the data display during an assay (e.g. changing the display from "Rate" to "Level" mode) can redirect a significant amount of computer resources away from data acquisition, and in some instances may result in data loss.
- **Do not modify the Windows OS language setting.**  
The Windows Operating System language is set to English and should not be modified. Changing this setting will significantly impact software functionality.
- **Close any open applications before starting an assay**  
Do not run applications in the background during an assay (e.g., Microsoft Excel, web browser, etc.) as this limits available PC memory for XF Pro Controller software.

# Claiming your Agilent Seahorse XF Discovery License using XF Pro Controller software

## Adding the XF Discovery License to your XF Pro Controller software

### About the XF Discovery License

This section provides information on how to obtain and install the XF Discovery license. The XF Discovery license is a single-use node license that is claimed through Agilent SubscribeNet and installed within the XF Pro Controller software application. Data files generated by an XF Pro Analyzer with the XF Discovery license will have access to important & valuable data analysis features in Wave Pro software and Seahorse Analytics, such as plotting data in a dose response curve, analysis of multiple result files, and more. Seahorse XF Pro Analyzers with the XF Discovery license also will have access to the automation service, enabling you to automate the XF assay workflow, from template selection to swapping plates & executing an assay.

A 30-day trial of the XF Discovery license is built-in to the XF Pro Controller software, which can be activated at your discretion at any time (**Figure 67**). During this trial period, every assay result file (XFLR) generated by the XF Pro Analyzer will have access to enhanced data analysis features in Seahorse Wave Pro software and Seahorse Analytics (web-based) software. To continue using these features after the 30-day trial, you must purchase and install the core XF Discovery license file.

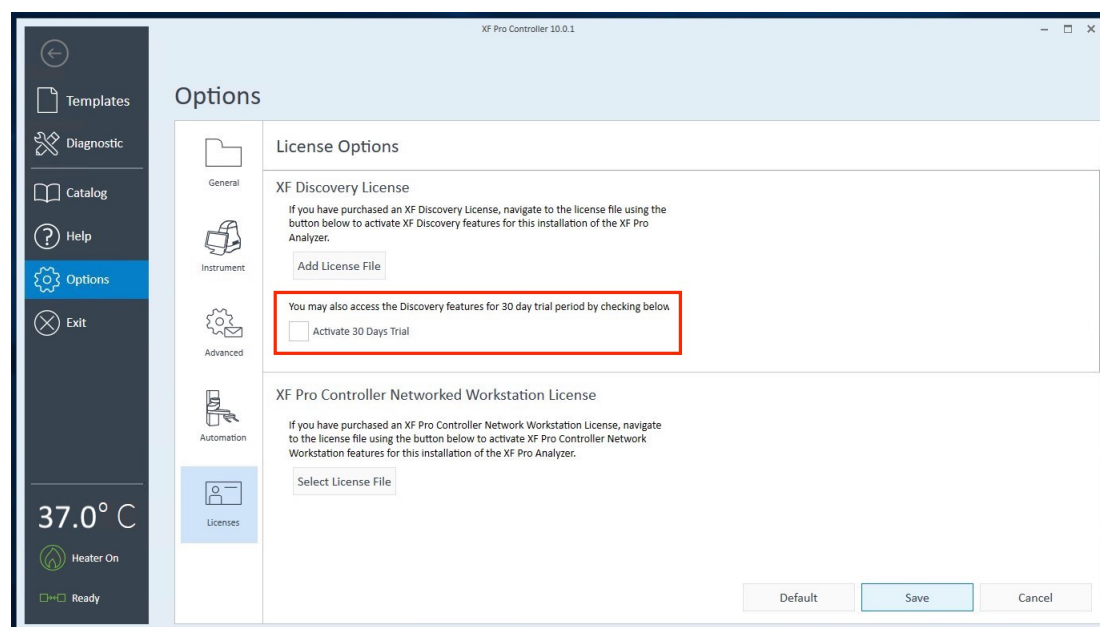


Figure 67. SWLIC-F0

A license file will contain your XF Discovery software license. This license file is installed on the XF Pro Controller computer within the XF Pro Controller software application. Once the XF Discovery license is installed on the XF Pro Controller, it is bound to this computer and cannot be moved to another XF Pro Controller computer.



### 3 Run Assay on the XF Pro Analyzer

#### Obtain a License with SubscribeNet

The most efficient way to manage and maintain your licensing is through the Internet. To generate, download, and install a final license for your product, you will need:

- A computer with internet access.
- The authorization code label provided in the lavender envelope containing your Software Entitlement Certificate.
- The URL for SubscribeNet from the Software Entitlement Certificate.

If you have not received a lavender envelope for your product, contact Agilent Cell Analysis technical support.

## Obtain a License with SubscribeNet

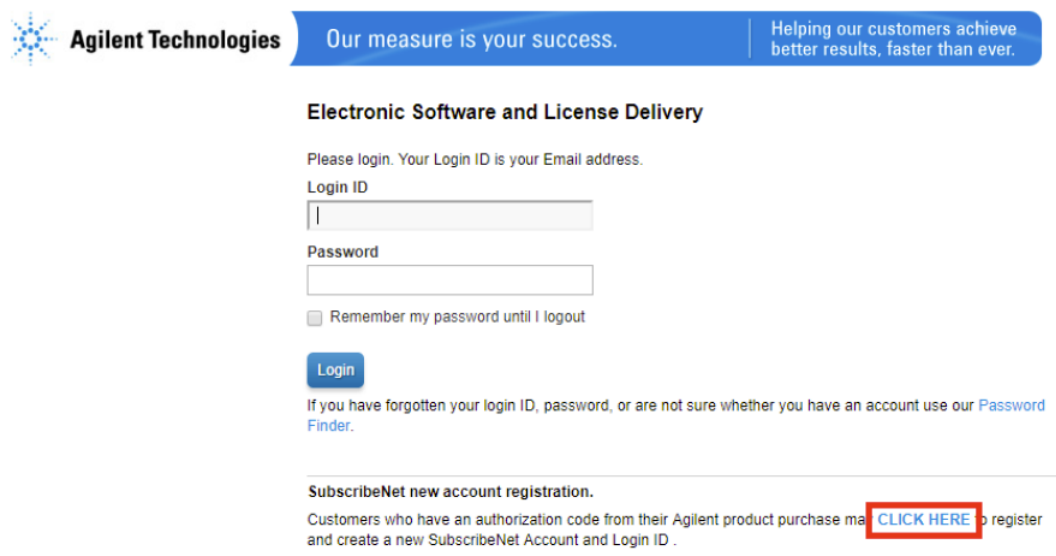
If you have internet access, use the following procedure to generate and download your license for your XF Pro system.

If you are a new user who has not registered with SubscribeNet, continue with the section **“New Users”** on page 61.

If you have registered with SubscribeNet, skip to the section **“Users registered with SubscribeNet”** on page 68.

### New Users

- 1 From a computer with Internet access, enter the URL provided in the Software Entitlement Certificate in an Internet browser: <https://agilent.subscribenet.com>
- 2 At the bottom of the login page, click **click here** to register (**Figure 68**).



**Agilent Technologies** Our measure is your success. Helping our customers achieve better results, faster than ever.

### Electronic Software and License Delivery

Please login. Your Login ID is your Email address.

Login ID

Password

☐ Remember my password until I logout

Login

If you have forgotten your login ID, password, or are not sure whether you have an account use our [Password Finder](#).

---

**SubscribeNet new account registration.**  
Customers who have an authorization code from their Agilent product purchase may [CLICK HERE](#) to register and create a new SubscribeNet Account and Login ID.

Figure 68. SWLIC-F1

- 3 On the registration page, enter the authorization code from the label and complete the profile information (**Figure 69**). Required fields are marked with an asterisk \*

### 3 Run Assay on the XF Pro Analyzer

#### Obtain a License with SubscribeNet

The email address you enter will become your login ID.

The image shows a web form for Agilent Technologies. At the top, there is a blue header with the Agilent logo and the text "Our measure is your success." and "Helping our customers achieve better results, faster than ever." Below this is the section "Electronic Software and License Delivery". Underneath is the "Registration Information" section. A paragraph explains that registration information is used to create a new SubscribeNet Account and Login ID (email address) for the Account. An authorization code received from the product purchase is required. Below this is the "Important Email Notes" section with two bullet points: "Please ensure the email address is entered correctly or you will not receive a welcome email with your Login ID and Password." and "If the email entered has already been used for an account Login ID, you will not receive a welcome email. The new account will display as an additional selection after you log in to SubscribeNet." Below the notes is a form with various fields. A note states "\* Indicates a required field." The fields are: Authorization Code\*, Email\*, Re-enter Email to Verify\*, Company\*, Department\*, First Name\*, Last Name\*, Job Title, Phone\*, Address 1\*, Address 2, Address 3, City\*, State/Province\* (dropdown menu), Postal Code, and Country\* (dropdown menu). The "Submit" button is highlighted with a red box. At the bottom, there are links for Privacy Statement, Terms of Use, Agilent Home, and a copyright notice for Agilent 2000-2018, along with a link to the EU Safe Harbor Notice.

Agilent Technologies

Our measure is your success.

Helping our customers achieve better results, faster than ever.

### Electronic Software and License Delivery

#### Registration Information

Registration information is used to create a new SubscribeNet Account and Login ID (email address) for the Account. An authorization code you received from your product purchase is required.

#### Important Email Notes

- Please ensure the email address is entered correctly or you will not receive a welcome email with your Login ID and Password.
- If the email entered has already been used for an account Login ID, you will not receive a welcome email. The new account will display as an additional selection after you log in to SubscribeNet.

\* Indicates a required field.

Authorization Code:\*

Email:\*

Re-enter Email to Verify:\*

Company:\*

Department:\*

First Name:\*

Last Name:\*

Job Title:

Phone:\*

Address 1:\*

Address 2:

Address 3:

City:\*

State/Province:\*

Postal Code:

Country:\*

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Figure 69. SWLIC-F2

- 4 Click **Submit**. The system will generate and display an account name for you. SubscribeNet will send a welcome email with your login ID and password.
- 5 Log in to SubscribeNet using your login ID and password. Once you log in, you can use the online user manual link for help with any questions you have.

### 3 Run Assay on the XF Pro Analyzer

#### Obtain a License with SubscribeNet

6 From the Product Information view (**Figure 70**), click **Seahorse Licenses**.

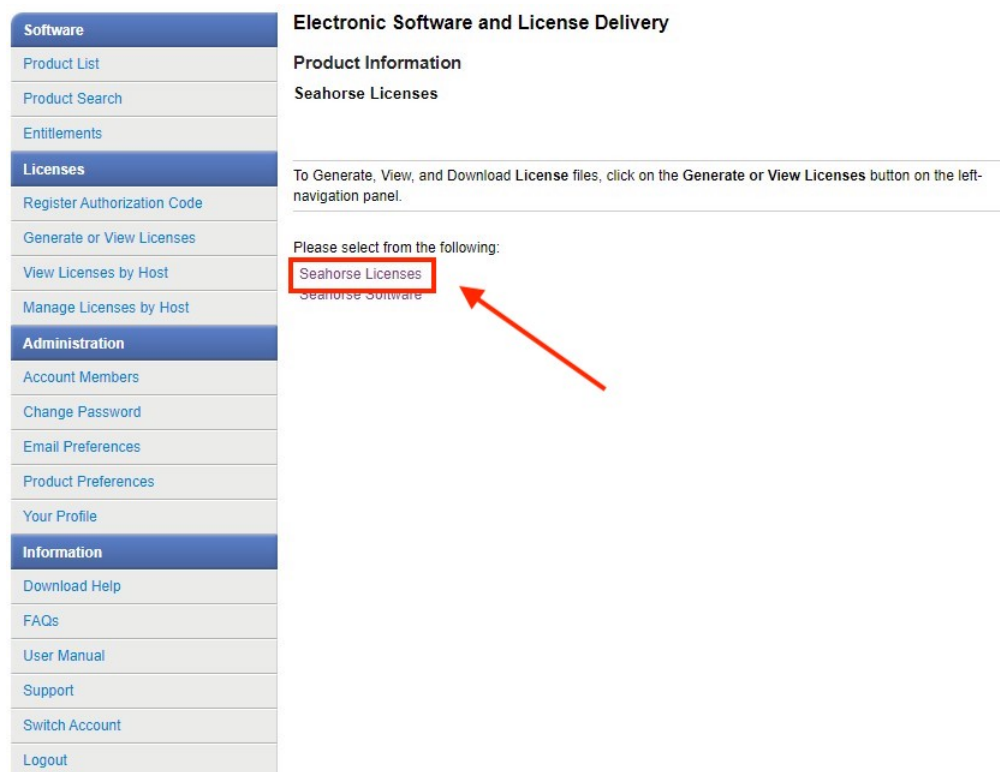


Figure 70. SWLIC-F3

### 3 Run Assay on the XF Pro Analyzer

#### Obtain a License with SubscribeNet

7 Click **Seahorse XF Pro Discovery License** (Figure 71).

**Software**

- Product List
- Product Search
- Entitlements
- Licenses**
- Register Authorization Code
- Generate or View Licenses
- View Licenses by Host
- Manage Licenses by Host

**Administration**

- Account Members
- Change Password
- Email Preferences
- Product Preferences
- Your Profile

**Information**

- Download Help
- FAQs
- User Manual
- Support
- Switch Account
- Logout

**Electronic Software and License Delivery**

**Product Information**

**Seahorse Licenses**

To Generate, View, and Download License files, click on the **Generate or View Licenses** button on the left-navigation panel.

Select a version.

**New Versions** Previous Versions

Version	Description	Date Available
1.0	Seahorse XF Pro Controller NWS Software License	Jun 21, 2021
1.0	Seahorse XF Pro Discovery License	Jun 21, 2021
1.0	Seahorse XF Cell Counting Software License	Apr 4, 2018

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Figure 71. SWLIC-F4

### 3 Run Assay on the XF Pro Analyzer

#### Obtain a License with SubscribeNet

8 Click **Register Authorization Code** in the left-navigation panel (**Figure 72**).

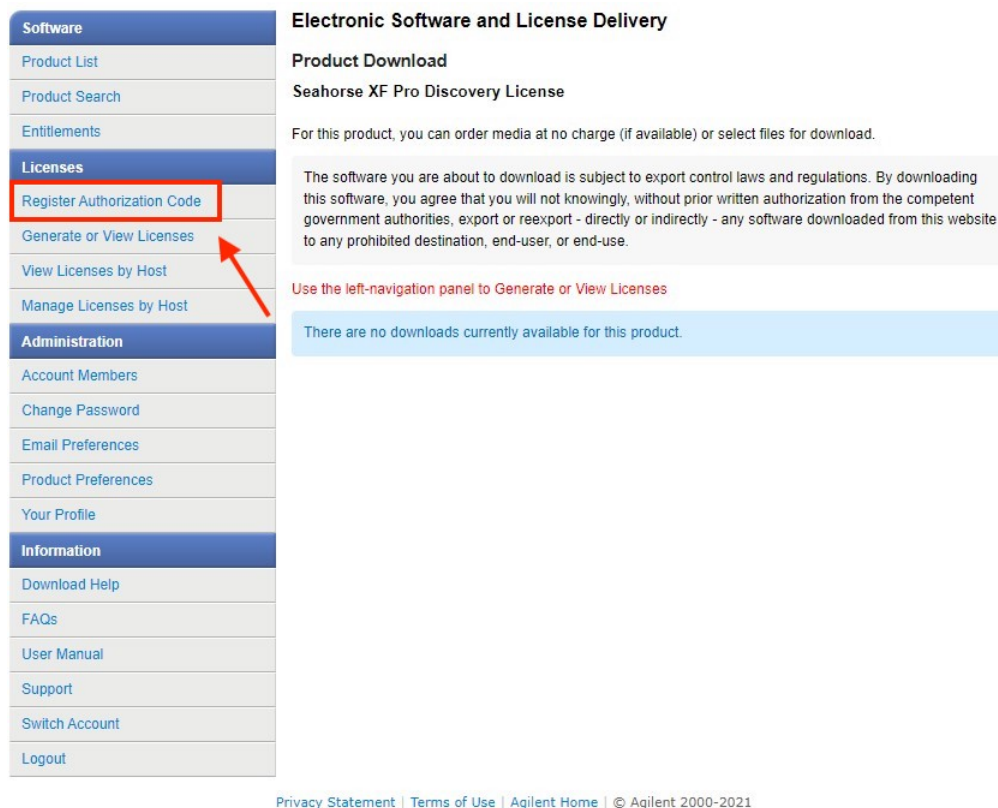


Figure 72. SWLIC-F5

9 A screen will appear prompting for an Authorization Code (**Figure 73**), which can be found on the authorization code label provided in the lavender envelope containing your Software Entitlement Certificate. Enter the code and click **Submit**.

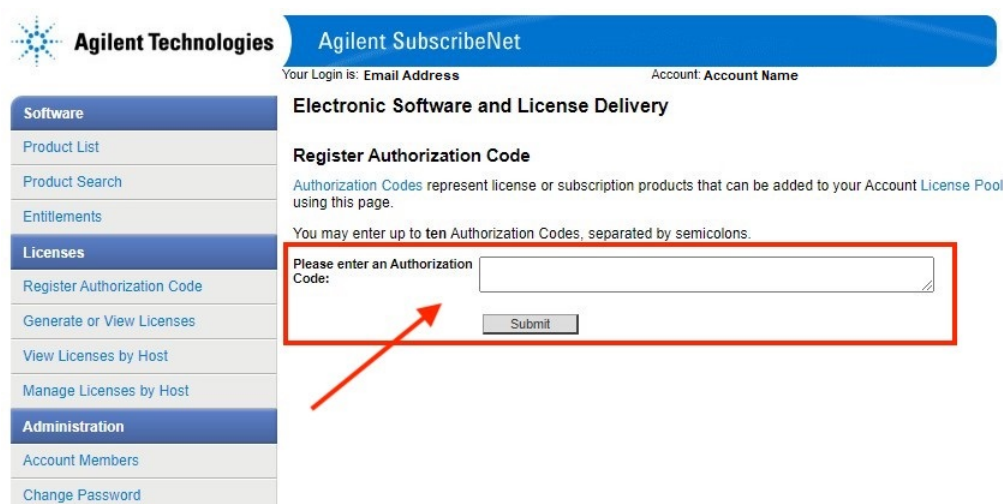



Figure 73. SWLIC-F6

3 Run Assay on the XF Pro Analyzer  
Obtain a License with SubscribeNet

10 The next dialog prompts the generation of the license. Check the box next to the XF Discovery license and click **Generate** (Figure 74).

 **Agilent Technologies**

Agilent SubscribeNet

Your Login is: Email Address

Account: Account Name

Software

Product List

Product Search

Entitlements

Licenses

Register Authorization Code

Generate or View Licenses

View Licenses by Host

Manage Licenses by Host

Administration

Account Members

Change Password

Email Preferences

Product Preferences

Your Profile

Information

Download Help

FAQs

User Manual

Support

Switch Account

Logout

Electronic Software and License Delivery

Generate or View Licenses

For licenses in your License Pool, you may:

- Select one or more licenses to **Generate** by checking the **left** box or boxes.
- Select one or more generated licenses to **View** by clicking the 'View Details' link, and then checking the **right** box or boxes.

License Returns

Generated licenses may be returned to the License Pool by clicking the 'View Details' link, and then clicking the 'Return' button.

More Help...

Entitlement Number:

Entitlement Date:

Generate

View

Seahorse XF Pro Discovery Software

Total Number of Licenses: 1

☐

Version

1.0

Description

Seahorse XF Pro Discovery License

1 Available

Generate

View

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Figure 74. SWLIC-F7

### 3 Run Assay on the XF Pro Analyzer

#### Obtain a License with SubscribeNet

- 11 On the next dialog, you must enter the XF Pro Controller computer MAC address. You can find this information on the Help view in XF Pro Controller software. Once you have entered the Host ID MAC Address, click **Generate** (Figure 75).

**Agilent Technologies** Agilent SubscribeNet

Your Login is: Email Address Account: Account Name

**Electronic Software and License Delivery**

**Generate Licenses**

For your selected license, enter the Host ID information:

This is the twelve-digit MAC Address of your computer. It may be entered as:

- twelve digits
- twelve digits separated by a colon (nn:nn:nn:nn:nn:nn)
- twelve digits separated by a hyphen (nn-nn-nn-nn-nn-nn)

Version	Description	Number of Licenses Available
1.0	Seahorse XF Pro Controller NWS Software License	1

Host ID MAC Address

**Generate**

Figure 75. SWLIC-F8

### 3 Run Assay on the XF Pro Analyzer

#### Install your XF Discovery License

- 12 When SubscribeNet generates the license, you will automatically see the View Licenses by Host dialog, click **Download License File** (Figure 76). Save the license file to your computer and to a backup location, such as a portable storage device.

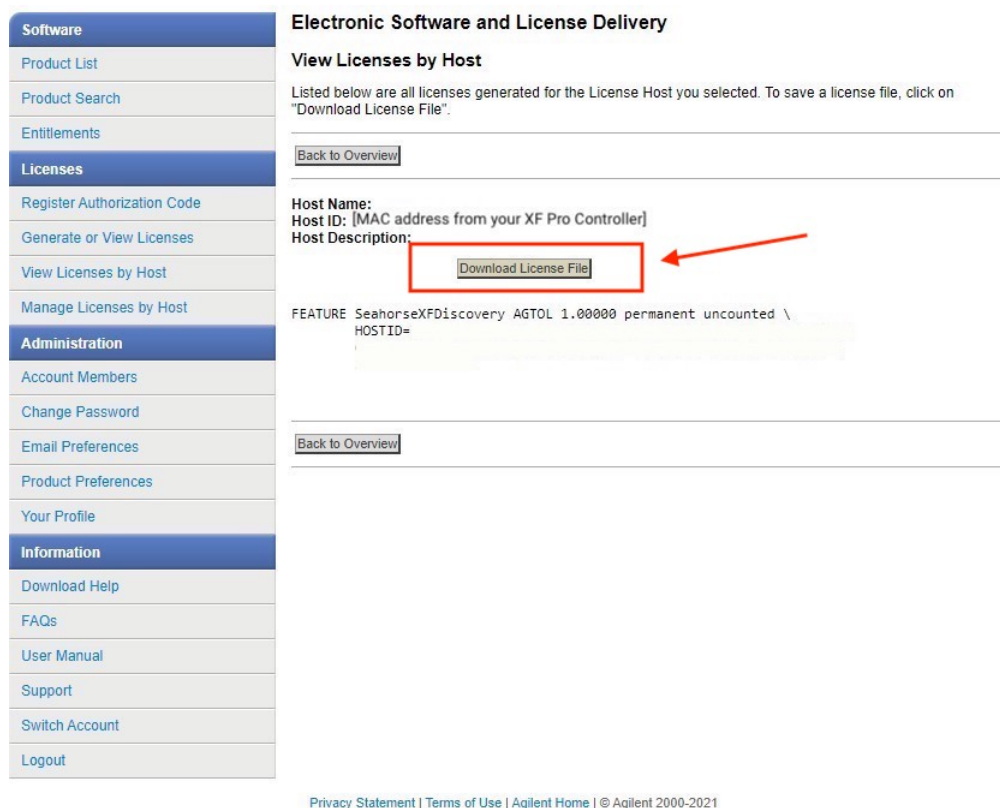


Figure 76. SWLIC-F9

Save your SubscribeNet login ID and password for future use of the Agilent SubscribeNet site.

#### Users registered with SubscribeNet

- 1 Login to SubscribeNet with your e-mail address and password.
- 2 From the SubscribeNet navigation pane, select **Register Authorization Code**. This will allow you to enter your new authorization code and make available the new license entitlements.
- 3 Follow steps 5 through 12 in the previous procedure, New Users, to generate or view your new licenses.

## Install your XF Discovery License

Once you have downloaded your XF Discovery license file, you must add the license file to your XF Pro Controller computer using XF Pro Controller software:

- 1 Create a folder called "licenses" on the C: drive
- 2 Copy your downloaded XF Discovery license file to this folder.
- 3 Start the XF Pro Controller software application.



### 3 Run Assay on the XF Pro Analyzer

#### Install your XF Discovery License

- 4 Using the left-side navigation, click **Options > Licenses** (Figure 77).

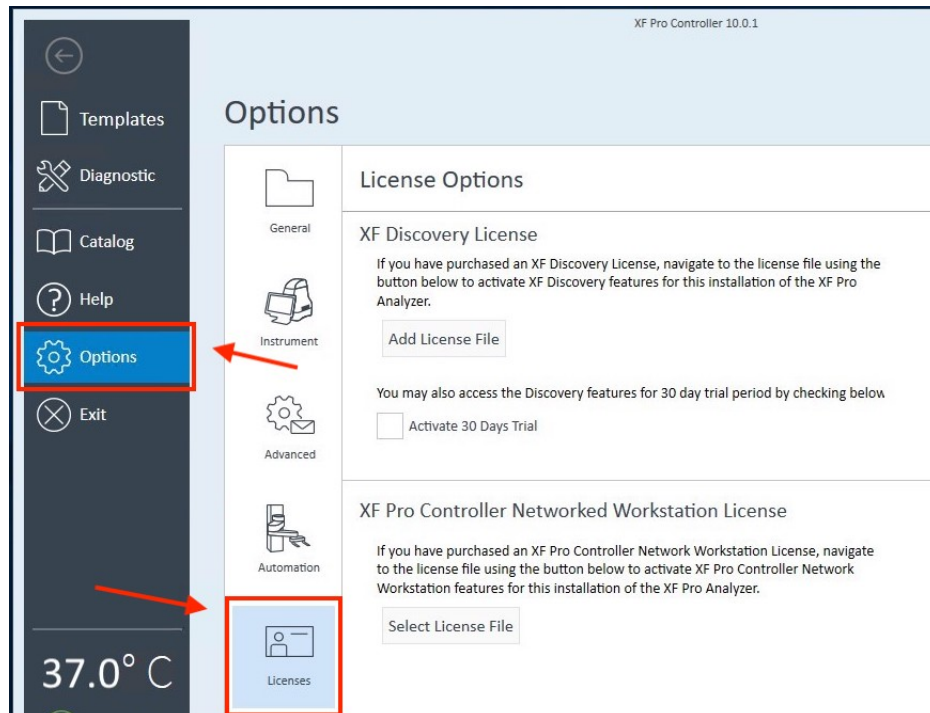


Figure 77. SWLIC-F10

- 5 Click the **Add License File** button (Figure 78).

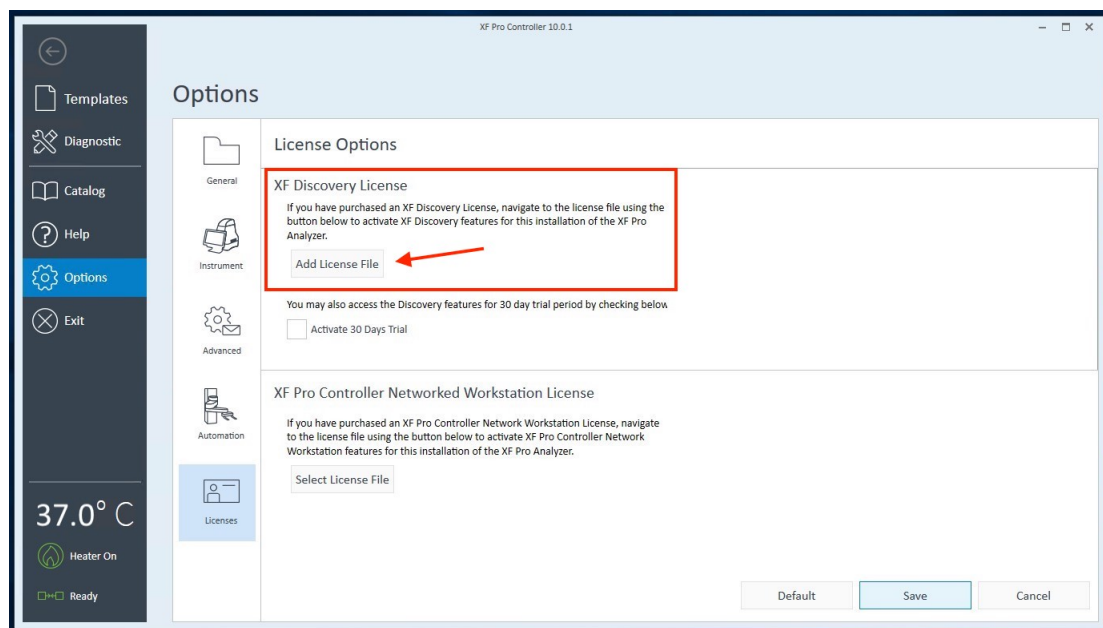


Figure 78. SWLIC-F11

- 6 Using the dialog that appears, navigate to the folder containing your XF Discovery license file, select your file and click **Open**.

### 3 Run Assay on the XF Pro Analyzer

#### Install your XF Discovery License

- 7 Now on the Help view in XF Pro Controller software you will see the license file name displayed (**Figure 79**). Click **Save** on the **Licenses > Options** view. XF Pro Controller software will automatically restart.

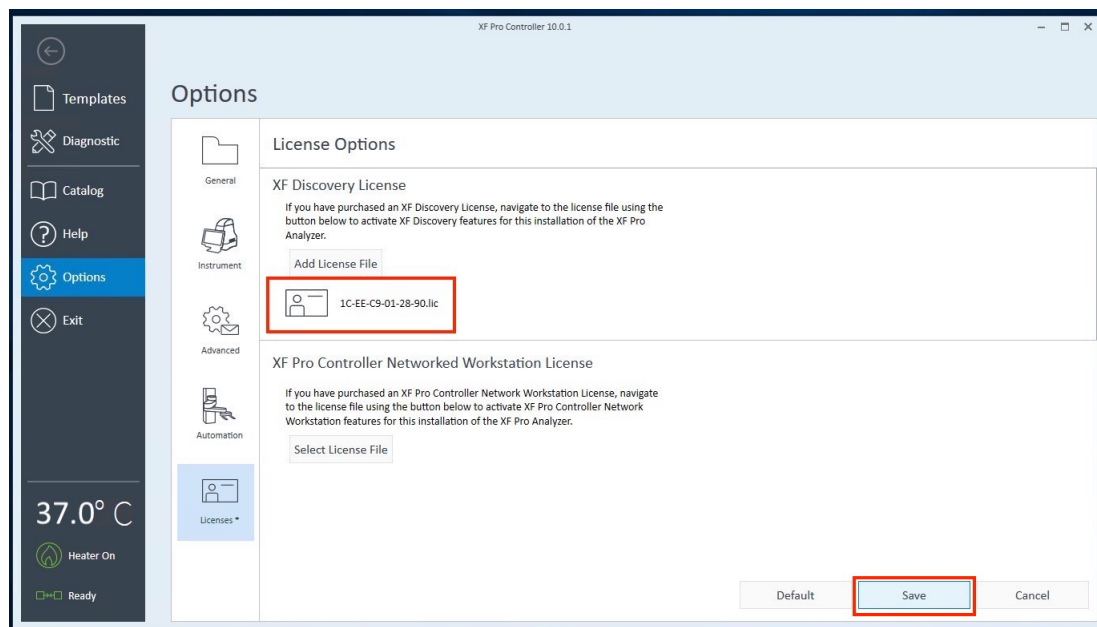


Figure 79. SWLIC-F12

- 8 Confirm the XF Discovery license is accepted following software restart. Click **Options > Licenses** to verify the XF Discovery license file name is displayed (**Figure 80**).

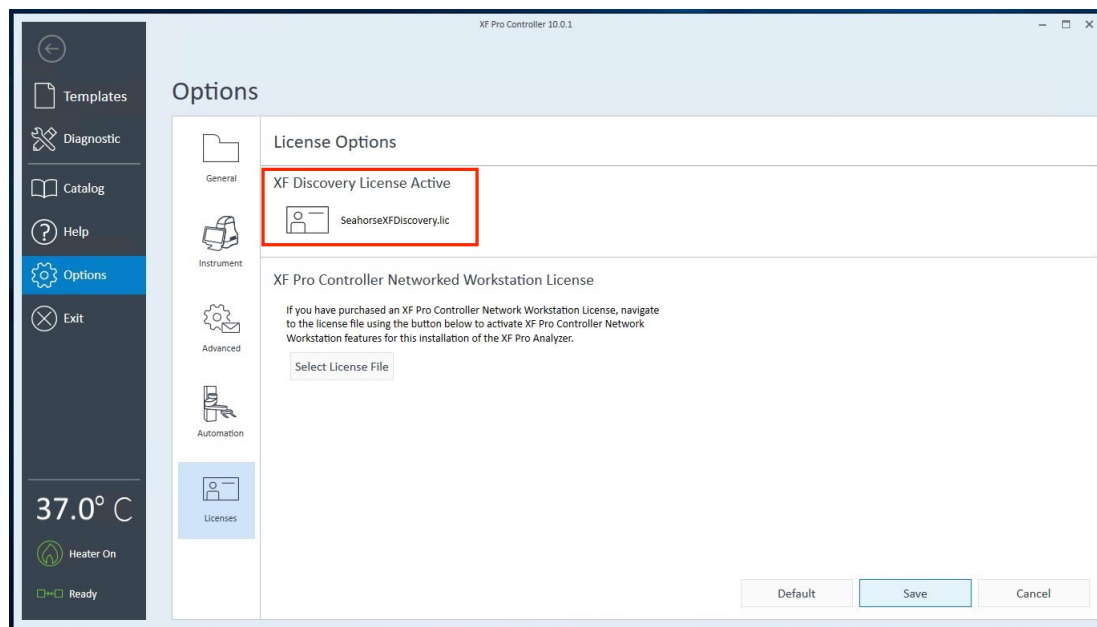
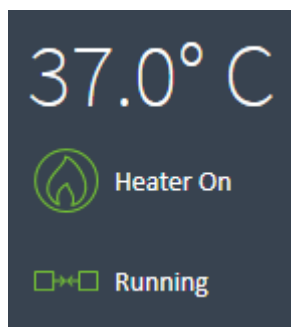


Figure 80. SWLIC-F13

## XF Pro Controller Widgets

The Widget icons are located on the lower left side of XF Pro Controller software, and display the analyzer status, current temperature, controls to eject/insert the tray and raise/lower probes.



Status widget: Connection status between the XF Pro Controller computer, XF Pro Controller software, and the XF Pro Analyzer.

Temperature widget: Current tray temperature and heater status display.

## Seahorse Assays at non-37 °C temperatures

Seahorse XF Pro Analyzers have been validated to deliver desired target temperatures in the range of 16-42 °C provided the ambient room temperature is 8-20 °C below the target temperature, and in the validated operational room temperature range of 4-30 °C. To understand the relationship between the sample temperature and ambient temperature, see [Figure 81](#) on page 72 for the temperature chart.

## Required operational and assay guidelines

- For all non-37 °C operation, the XF Pro Analyzer must equilibrate overnight in required ambient temperature.
- If required to set up the XF Pro Analyzer in cold room, avoid direct fan sources. (See [Figure 81](#) on page 72.)
- For all non-37 °C operation, the tray heater must remain ON, do NOT turn the tray heater OFF.
- For assay temperatures below 30 °C, hydrate the Sensor Cartridge in the dark at room temperature.
- Prior to starting an assay, an additional 30 minutes of precalibration equilibration time has been added to ensure temperature stability.

3 Run Assay on the XF Pro Analyzer  
Required operational and assay guidelines

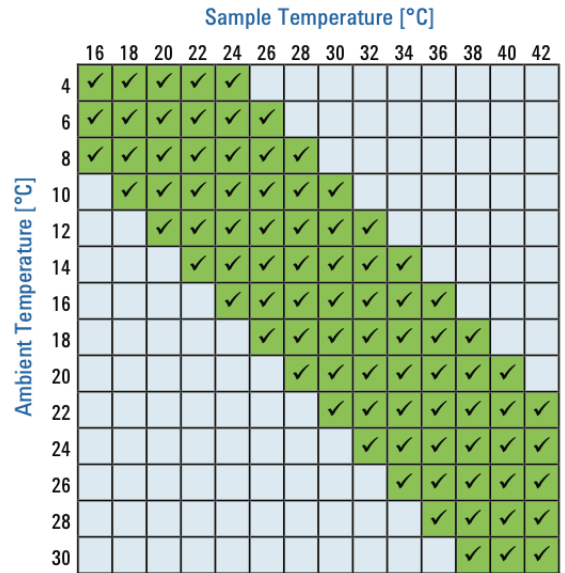


Figure 81. Temperature chart

To adjust the Target Temperature (set point) using the up/down arrows, click on the **Temperature Widget** to display the Tray Temperature window.

(See [Figure 82](#) and [Figure 83](#) on [page 72](#).) Ensure your ambient conditions support the desired target temperature (12-20 °C above ambient). Refer to the temperature chart shown in [Figure 81](#) on page 72.

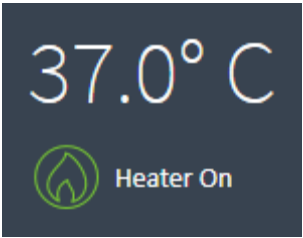


Figure 82. Temperature widget

NOTE

Changing the Target Temperature requires OVERNIGHT equilibration to the new set point.

### 3 Run Assay on the XF Pro Analyzer

#### Set alarm (Temperature tolerance range)

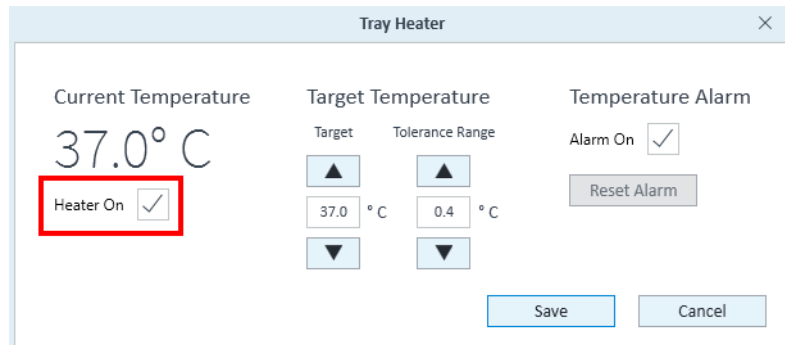


Figure 83. Edit temperature settings window

Other Temperature Widget functions are:

- Turn the heater **ON/OFF**.
- Set the tolerance range for temperature fluctuation. If the temperature is above or below the acceptable tolerance range from the temperature set point, the Temperature Widget will change color (see [Figure 84](#)), and the Status Indicator light (top of the XF Pro Analyzer) will change from blue to amber. If configured, XF Pro Controller software can automatically notify users (via email) if temperature warnings are triggered. This feature requires an active internet connection on the XF Pro Controller (computer).

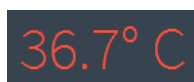


Figure 84. Temperature out-of-range widget status

To save any changes on the Tray Temperature window, click **Save**.

## Set alarm (Temperature tolerance range)

To set the alarm:

- 1 Check the **Alarm On** box in the Tray Temperature window. (See [Figure 85](#).)

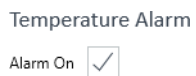


Figure 85. Temperature Alarm

- 2 Click **Save**.

Disable the alarm by unchecking the **Alarm On** box, then click **Save**. If the Tray Temperature exceeds the Tolerance Range and the alarm is activated, click **Reset Alarm** to acknowledge and reset the Tray Temperature alarm.

To ensure the Tray Temperature starts within the Tolerance Range, check the current temperature of the XF Pro Analyzer before beginning an assay. For any suspected temperature issues or unexpected temperature fluctuations, contact **Agilent Cell Analysis Technical Support**.

## XF Pro Status Indicator

The status indicator light, located at the top of the XF Pro Analyzer, will change to an amber color when the XF Pro Controller software and Analyzer are not connected, or user interaction is required, such as:

- To load a Sensor Cartridge or Cell Plate
- To remove a used Sensor Cartridge and/or Cell Plate
- To accept or cancel an assay if one or more wells did not calibrate properly after Calibration
- Any errors that can occur during the run, such as barcode read errors for the Sensor Cartridge,

The status indicator light will change to red if any errors occur, such as barcode read errors for the Sensor Cartridge, Cell Plate, or a protocol error.

## Barcode Errors

The XF Pro Analyzer reads and records the Cell Plate and Sensor Cartridge barcodes before beginning an assay. A Barcode Read error will be displayed on the rare occasion the barcode cannot be read. (See [Figure 86](#).) Contact [Agilent Cell Analysis Technical Support](#) to assist with resolving this error and to start the assay.

### Cartridge barcode read failure

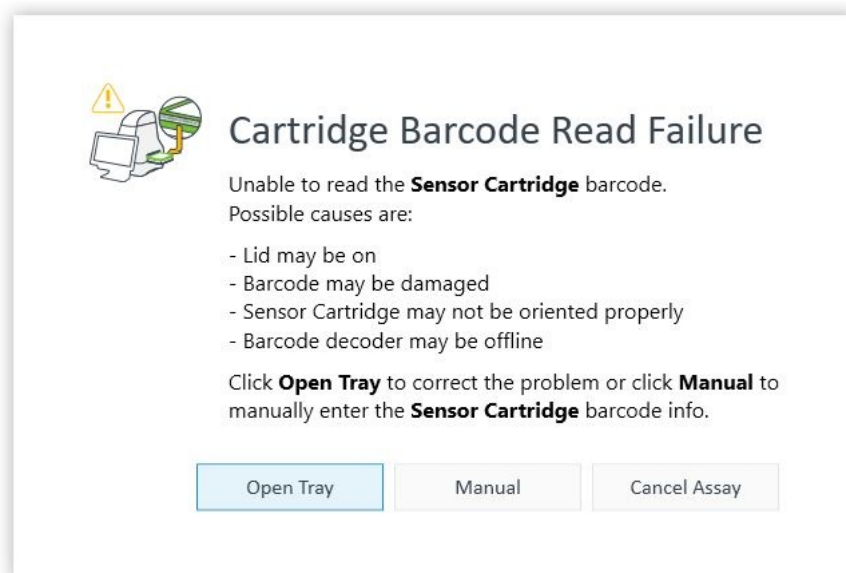


Figure 86. Barcode read failure

For any Sensor Cartridge barcode read errors, XF Pro Controller software will display the message shown in [Figure 86](#) and three corrective actions:

- **Open Tray:** Eject the Sensor Cartridge to inspect barcode quality or to reverse the Sensor Cartridge.
- **Manual:** Manually input the Sensor Cartridge barcode information. Contact [Agilent Cell Analysis Technical Support](#) for this step.
- **Cancel Assay:** Cancel the assay.

### 3 Run Assay on the XF Pro Analyzer

#### Cartridge barcode read failure

#### Manually enter sensor cartridge barcode

- 1 To display the Cartridge Barcode window, click Manual. (See [Figure 86](#))
- 2 Call the appropriate regional Agilent Cell Analysis Technical Support telephone number to assist with entering the Sensor Cartridge barcode info for each field on the form. (See [Figure 87](#).)

### Cartridge Barcode Manual Entry

Open tray to eject the cartridge and manually enter the Serial and Lot numbers into the fields below. If you have used this cartridge before, the remaining fields will autofill and you can return the sensor cartridge to the instrument. If validation isn't possible you will need to contact Agilent Cell Analysis Technical Support using the telephone numbers below.

**Global/United States:** +1 719 528 7500  
**United States (Toll Free):** +1 800 227 9770  
**UK, Ireland, France, Germany, Denmark, Netherlands (Toll Free):** 00 800 345 347 00  
**China (Toll Free Landline):** 800 820 3278  
**China (Toll Free Cell Phone):** 400 820 3278  
**Any other country:** +45 8830 5500  
**Email:** cellanalysis.support@agilent.com

Lot Number	<input type="text"/>
Serial Number	<input type="text"/>
Buffer Factor	<input type="text"/>
O2_A	<input type="text"/>
O2_B	<input type="text"/>
PH_A	<input type="text"/>
PH_B	<input type="text"/>
PH_C	<input type="text"/>

Figure 87. Cartridge barcode manual entry form



## Cell plate barcode read failure



### Cell Plate Barcode Read Failure

Unable to read **Cell Plate** barcode. Click **Manual** to enter the barcode manually.



Figure 88. Cell Plate Barcode Read Failure

For any Cell Plate barcode read errors, XF Pro Controller software will display the message shown in **Figure 88** and two corrective actions:

- **Manual:** Manually input the Cell Plate barcode info.
- **Cancel Assay:** Cancel the assay.

### Manually enter cell plate barcode information

#### Cell Plate Manual Barcode Entry

Barcode

Figure 89. Cell Plate Manual Barcode Entry

- 1 Click the Tray Control Widget.
- 2 To eject the Cell Plate, click **Open Tray**. (See **Figure 161** on page 145.)
- 3 The Cell Plate barcode is located on the side of the plate. Write down the barcode information.
- 4 Click **Close Tray**.
- 5 On the Cell Plate Manual Barcode Entry window, enter the Cell Plate barcode, and click **Accept**. (See **Figure 89**.)

# Analyzing Assay Results using Wave Pro

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The primary purpose of Wave Pro software is for data review & analysis of assay result files generated by Seahorse XF Pro and XF HS Mini Analyzers.

## Assay Result Files

Assay result files (.xflr) are the data files generated by XF Pro Analyzers and XF HS Mini Analyzers after completing an XF assay. Types of data is acquired during an XF assay include:

- Rate data (OCR & ECAR)
- Level data (mmHg or mpH)
- Calibration results
- Consumable information from the barcodes on the cell plate and sensor cartridge used in the assay

In addition, any details added to the assay template file about the experiment, such as groups, compound, pretreatments, is transferred and saved in the assay result file. Most of this information can be edited using the **Modify** function. For more information, see **“Modifying Assay Result Files”** on page 111.

After completing an XF assay, save the result file and transfer to your PC for data review and analysis using Wave Pro software (or Agilent Seahorse analytics). You can transfer your data files using a USB flash drive (or network directory).

When you open an assay result file for the first time, you will see the **Quick View**. If you opened a previously analyzed result file, you will see the last-modified or viewed analysis view. Each analysis view can be added to an assay result file using the **Add View** button. You can add multiple analysis views to your result file, there is no limit. The six analysis views in Wave software are:

- Quick View
- Overview
- OCR vs. ECAR
- Heat Map (requires XF Discovery license or an assay kit license)
- Dose Response (requires XF Discovery license or an assay kit license)
- Data

## Analysis View #1 - Quick View

**Quick View** is the default analysis view displayed when opening a new assay result file. Quick view simultaneously displays a kinetic graph of OCR vs Time, ECAR vs Time, and a scatter plot of OCR vs. ECAR. (See [Figure 90](#).)

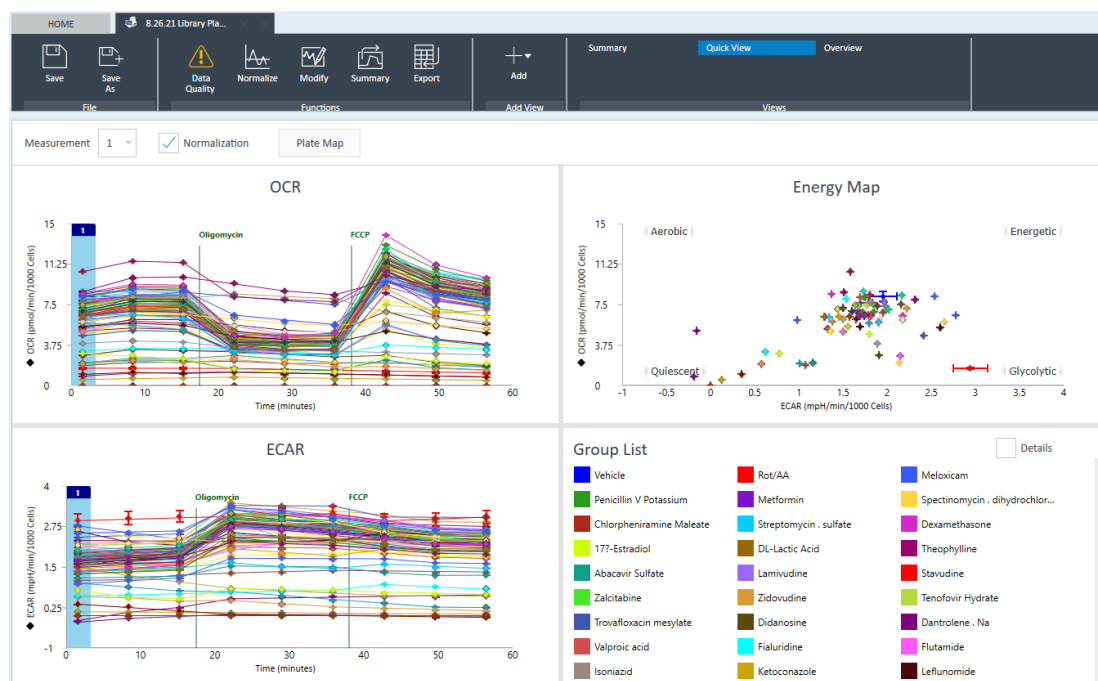


Figure 90. Quick view

## Analysis View #2 - Overview

**Overview** displays a kinetic graph of rate (**OCR**, **ECAR**, **PER**, or **PPR**) versus time. The selected rate is displayed on the y-axis, and **Time** is fixed on the x-axis.

Group statistics (average rate and error) for each measurement can be displayed by checking the **Details** box in the **Group List** below the kinetic graph. To display **Overview**, click **Add View** in the center of the ribbon menu, and select **Overview** from the list of views. (See [Figure 91](#).)

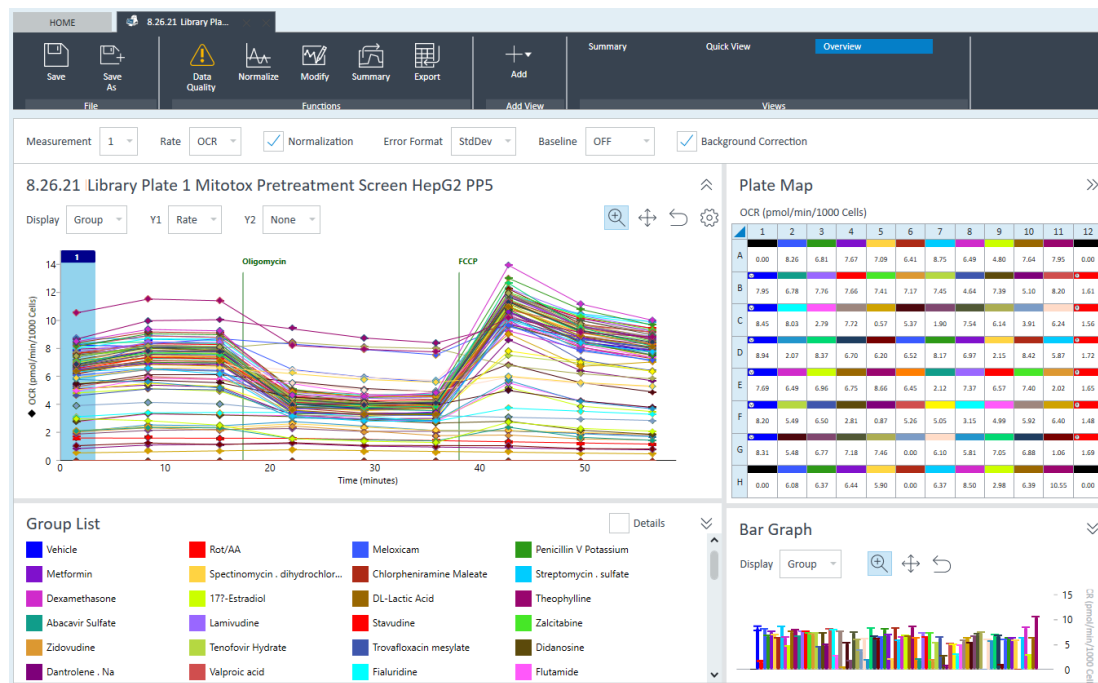


Figure 91. Overview

### NOTE

Add multiple Overview analysis views by repeating the process of Add View > Overview. This gives greater flexibility for tailoring your result data presentation to show specific groups, responses, or comparisons between groups.

## Analysis View #3 - OCR vs. ECAR

The **OCR vs. ECAR** view displays the **OCR** on the y-axis and (by default) **ECAR** on the x-axis. To display **OCR vs. ECAR**, click **Add View** in the upper-left region of the screen, and select **OCR vs. ECAR** from the drop-down menu. Use the rate drop-down menu to change the rate displayed on the x-axis to either **PER** or **PPR**. **OCR** is always displayed on the y-axis, and cannot be changed. (See [Figure 92](#).)

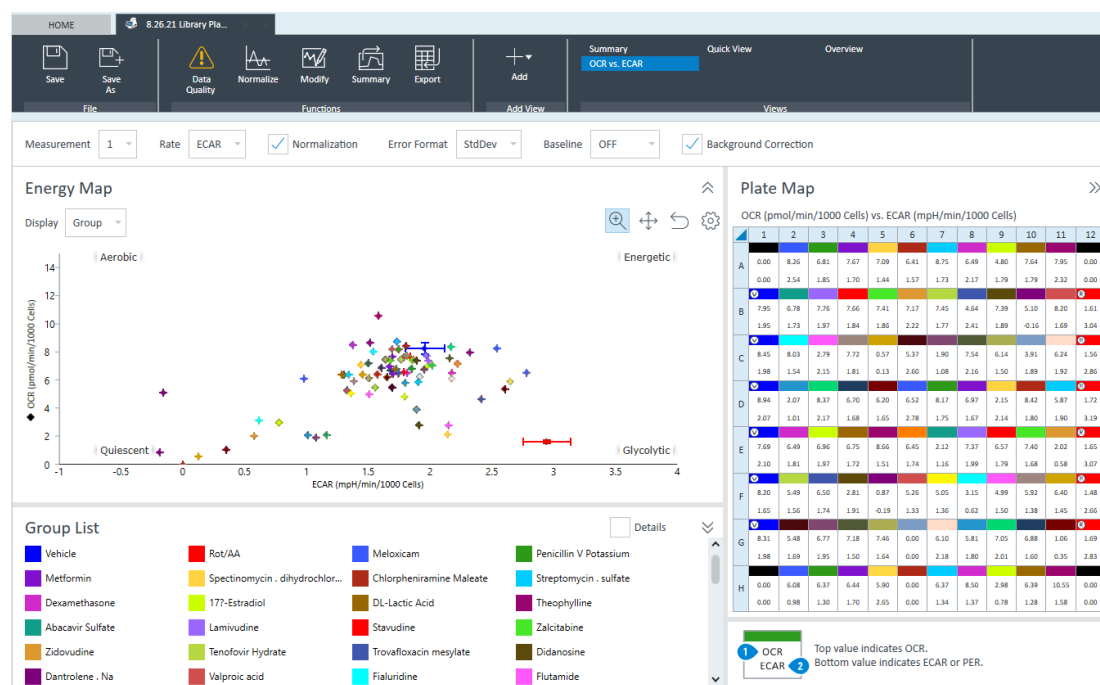


Figure 92. OCR vs. ECAR

### Graph title and Quadrant labels

The default graph title is **Energy Map**. (See [Figure 92](#).) The corners of the Energy Map have labels representing the relative bioenergetic state of the group(s) related to each other for the selected rate measurement. To modify the graph display, including the **Graph Title** and the **Quadrant Labels**, click the **Settings** cog. (See [Figure 93](#).)

Graph Title:

Show Energy Quadrant Labels: ☒

Figure 93. Graph display options

### Dual rate display on Plate Map

The **Plate Map** displays two rate measurements within each well (OCR vs. ECAR, OCR vs. PER, or OCR vs. PPR) as indicated by the legend below the Plate Map. (See [Figure 92](#).)

## Analysis View #4 - Heat Map

The **Heat Map** analysis view presents **OCR** or **ECAR** rate data illustrated in a colored plate map layout (**Figure 94**). The Heat Map analysis view presents rate data (OCR and ECAR) in a colored plate map layout. The color within each well depends the selected well's deviation from the plate average (for the selected rate measurement), enabling users to more easily identify differences in their data, or changes in functional metabolism that may not be obvious when viewing results in a different format, such as a kinetic graph or bar chart.

To display the Plate **Heat Map**, click **Add View** in the functions ribbon, and select **Heat Map** from the drop-down menu. Your XF Pro Analyzer must have an XF Discovery license (trial or full license) in order to add the Heat Map analysis view to your result file in Wave Pro. If you cannot add the Heat Map analysis view, this indicates the XF Pro Analyzer used to generate the data does not have an XF Discovery license.

To modify the rate measurement data presented in the heat map, use the Measurement drop-down menu and select the measurement to populate the plate map.

Use the **Rate** drop-down menu to change the rate displayed on the plate heat map to either **ECAR** or **OCR**. Heat values are represented by color shades. To adjust the Heat Map color distribution and tolerance, click the **Settings** cog. (**Figure 95**)

The selected measurement Rate Plot is displayed to the right, with time represented on the x-axis, and either **ECAR** or **OCR** rate type on the y-axis. Select the compounds to be displayed in the Rate Plot by clicking on compound samples in the Group List, or by clicking directly wells in the Heat Map Plate.

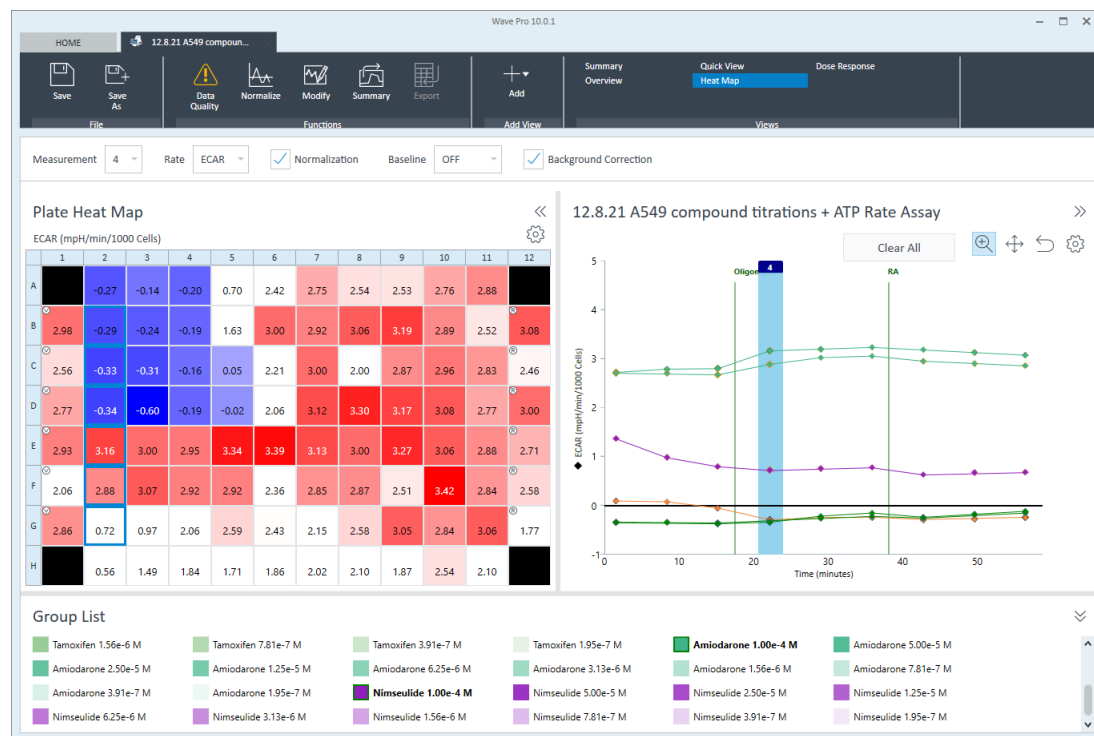


Figure 94. Heatmap view

## 4 Analyzing Assay Results using Wave Pro

### Analysis View #4 - Heat Map

Set the Color Tolerance from the drop down in the Hat Map settings. Lower tolerance percentage will accentuate value transition thresholds. You can also select the colors used for High and Low values.

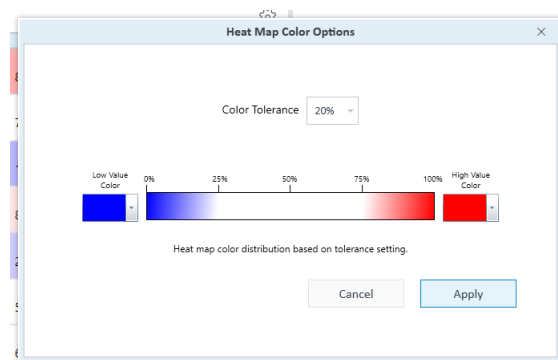


Figure 95. Heat Map Color Options



## Analysis View #5 - Dose Response

The **Dose Response** analysis view presents the **Dose Response curve plot**. To display **Dose Response**, click **Add View** in the functions ribbon, and select **Dose Response** from the drop-down menu. Your XF Pro Analyzer must have an XF Discovery license (trial or full license) in order to add the Dose Response analysis view to your result file. If you cannot add the Dose Response analysis view in Wave Pro, this indicates the XF Pro Analyzer used to generate the data does not have an XF Discovery license, and/or the template was not created using the titration setup function (see [page 41](#)).

The Dose Response analysis view presents rate data (OCR and ECAR) versus compound concentration for one or multiple titrates, in order to compare the effects of a compound at different doses on live cell metabolic activity.

To modify the rate measurement data presented in the dose response curve, use the Measurement drop-down menu. To display acidification data in the dose response, use the Rate drop-down menu. Use the rate drop-down menu to change the rate displayed as either **OCR** or **ECAR**.

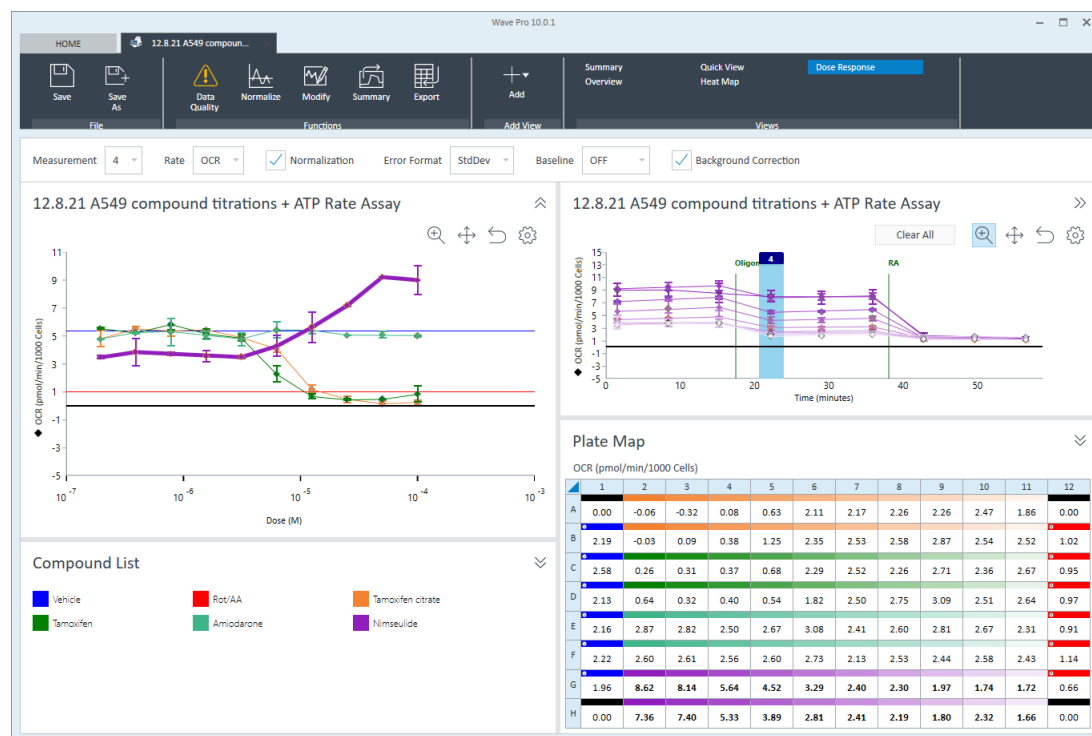


Figure 96. Dose Response view

The selected measurement Rate Plot is displayed to the right, with time represented on the x-axis, and either **ECAR** or **OCR** rate type on the y-axis. Select the measurement of interest from the Measurement drop-down. Select the compounds to be displayed in the Rate Plot by clicking on compounds in the **Compound List**. You can also show or hide individual titrations or by selecting them from the **Plate Map**.

#### 4 Analyzing Assay Results using Wave Pro

##### Analysis View #5 - Dose Response

To adjust the presentation of the dose response graph, click the Settings cog in the upper-right corner of the **Dose Response** display.

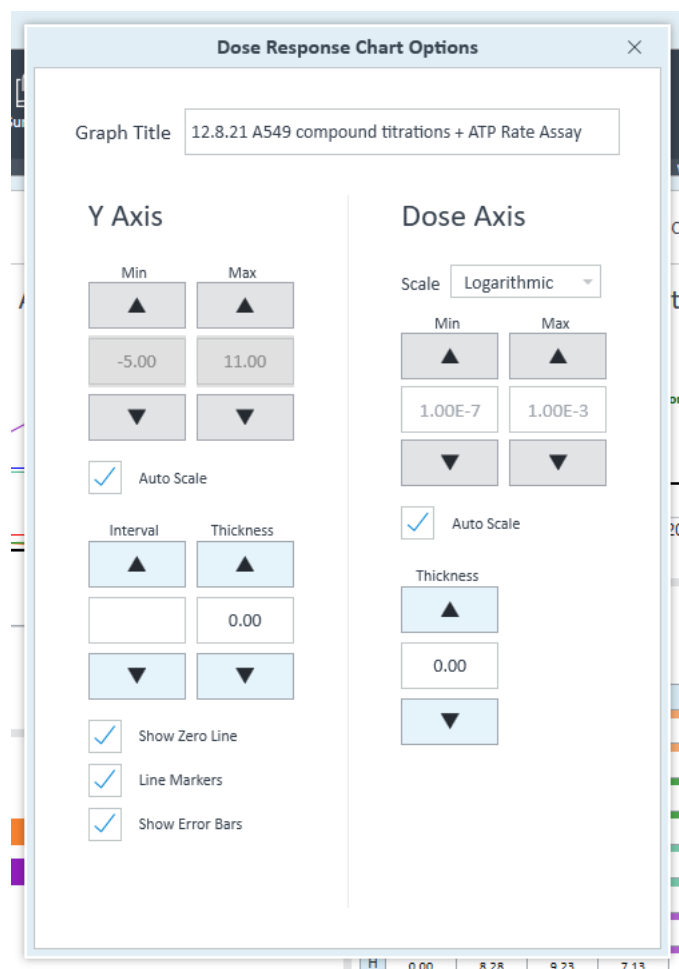


Figure 97. Dose Response chart options

Manually enter the scale range values for each axis or select **Auto Scale**. Properties such as line markers and line thickness can be adjusted from this option.

## Chart Types in Wave

### Kinetic graph (rate versus time)

A kinetic graph is the most common way to display rate data from XF Analyzers. (See [Figure 98](#).) The kinetic graph displays rate on the y-axis, and time on the x-axis. During an assay, data is acquired and plotted in real-time as a kinetic graph. Kinetic graphs are displayed in the Quick View and Overview analysis views.

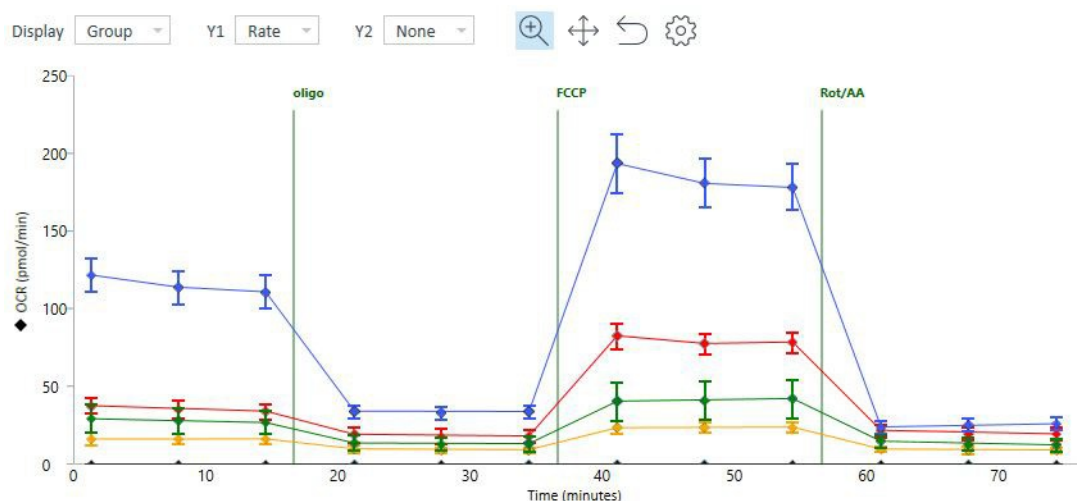


Figure 98. Kinetic graph

### Scatter plot (rate 1 versus rate 2)

Another common way to express result data is a scatter plot of OCR vs. ECAR, where OCR is plotted on the y-axis, and ECAR plotted on the x-axis. (See [Figure 51](#).) Data points are displayed on the OCR vs. ECAR scatter plot for a single rate measurement for each group in the assay. The OCR vs. ECAR graph is only available for analysis, not while running an XF assay.

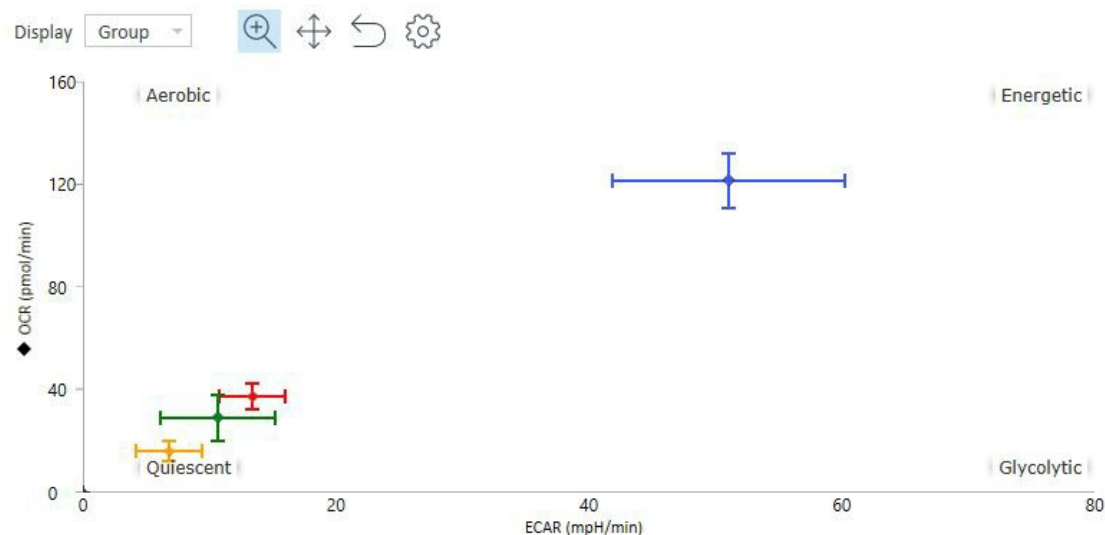


Figure 99. Scatter plot

## Heat Map

The Heat Map analysis view presents rate data (OCR and ECAR) Rate values, in a colored plate map layout. The color within each well depends the selected well's deviation from the plate average (for the selected rate measurement), enabling users to more easily identify differences in their data, or changes in functional metabolism that may not be obvious when viewing results in a different format, such as a kinetic graph or bar chart. Your XF Pro Analyzer must have an XF Discovery license (trial or full license) in order to add the Heat Map analysis view to your result file.

Plate Heat Map

OCR (pmol/min/1000 Cells)

	1	2	3	4	5	6	7	8	9	10	11	12
A		0.07	0.06	0.14	0.54	1.03	1.06	1.17	1.20	1.48	1.14	
B	1.12	0.10	0.17	0.41	0.90	1.17	1.25	1.23	1.44	1.31	1.41	1.02
C	1.54	0.30	0.32	0.29	0.66	1.18	1.30	1.26	1.25	1.41	1.44	0.97
D	1.13	0.62	0.34	0.36	0.52	1.05	1.16	1.28	1.51	1.47	1.40	0.93
E	1.16	1.11	1.18	1.02	1.30	1.46	1.44	1.36	1.50	1.41	1.12	0.94
F	1.27	1.03	1.01	1.08	1.25	1.52	1.15	1.30	1.41	1.48	1.35	0.98
G	1.07	2.13	1.74	1.47	1.53	1.58	1.26	1.37	1.22	1.48	1.24	0.70
H		1.59	1.47	1.34	1.33	1.12	1.26	1.40	1.12	1.87	1.27	

Figure 100. Heat Map chart

Toggle the **Normalization** option to view rate values with the scale factor applied.

In addition to the Plate Heat Map, the Heat Map view includes a Rate Plot in the form of a kinetic graph.

## Dose Response

The **Dose Response** analysis view presents rate data (OCR and ECAR) versus compound concentration for one or multiple titrates, in order to compare the effects of a compound at different doses on live cell metabolic activity.

Your XF Pro Analyzer must have an XF Discovery license (trial or full license) in order to add the Dose Response analysis view to your result file.

4 Analyzing Assay Results using Wave Pro  
Plate Map

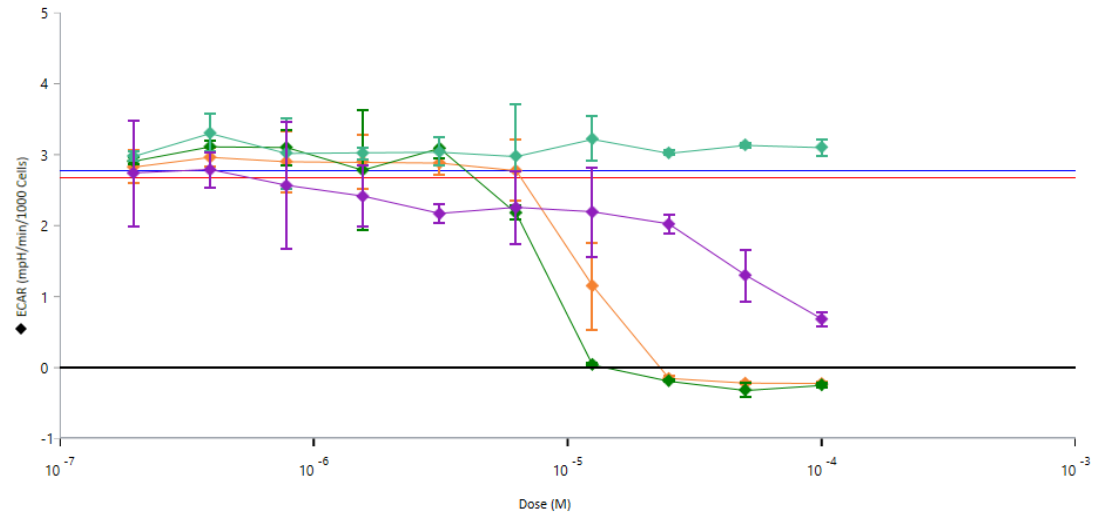


Figure 101. Dose Response view

## Plate Map

The Plate Map displays rate data for the selected rate measurement of each assay well. (See [Figure 102](#).) Plate Maps are typically found to the right of any graphed data, for example during runtime or as seen in the Overview analysis view. The Quick View has a button to display the Plate Map, which is hidden by default. The default rate measurement displayed on the Plate Map is rate measurement 1.

Plate Map >>

OCR (pmol/min/ug protein) vs. ECAR (mpH/min/ug protein)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.00 0.00	9.85 1.64	7.05 6.62	7.17 1.15	8.22 1.89	9.95 0.57	4.36 2.00	6.25 2.05	9.26 1.90	7.71 1.83	6.36 2.36	0.00 0.00
B	10.40 1.53	12.45 1.82	10.37 2.34	10.07 1.49	11.03 1.47	10.65 1.54	5.18 0.78	4.88 2.91	7.68 1.42	8.97 1.82	8.69 2.66	7.61 3.29
C	10.39 1.54	11.23 2.66	12.55 3.12	9.78 2.80	9.62 2.18	10.27 1.50	6.03 1.39	4.91 2.43	7.38 2.35	5.09 3.07	4.76 4.25	7.52 2.73
D	10.52 2.32	10.55 2.47	11.86 3.23	6.59 2.20	8.50 3.17	9.96 2.94	7.28 2.34	7.96 3.32	13.15 4.88	7.59 3.92	9.42 4.79	6.75 1.07
E	9.98 3.39	8.96 2.92	9.64 4.85	7.52 2.08	8.75 2.12	9.16 3.11	6.41 2.39	11.24 3.65	12.72 3.06	7.71 3.73	6.23 3.87	10.55 2.41
F	9.88 3.57	10.08 4.52	11.78 4.96	7.45 2.89	13.40 3.73	8.40 3.73	6.98 4.10	6.38 0.90	8.72 0.94	5.12 1.39	6.94 4.40	10.77 0.92
G	10.47 5.14	11.21 4.76	10.97 5.00	6.96 2.95	10.57 3.01	7.52 3.51	5.93 3.06	6.68 1.81	10.63 4.16	7.37 3.91	7.10 4.99	8.47 3.30
H	0.00 0.00	9.08 3.75	12.37 5.52	9.68 4.00	10.43 3.26	9.28 3.69	9.29 4.97	5.06 2.41	6.04 3.53	5.90 3.33	5.87 2.46	0.00 0.00

Figure 102. Plate map

## 4 Analyzing Assay Results using Wave Pro Plate Map

To change the measurement displayed in the Plate Map, use the Measurement drop-down above the kinetic graph or scatter plot. (See [Figure 103](#).) The Rate Highlight tool on the kinetic graph will move to the selected rate. Hide the Rate Highlight tool by clicking **Options** above the kinetic graph/scatter plot, and uncheck **Show Rate Highlight**.

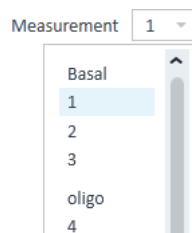


Figure 103. Rate measurement drop-down menu

### Flag wells on the plate map

Right-click a well on the plate map to display the add/remove flag function. (See [Figure 104](#).) Flags are an analysis feature only and can be added from any analysis view (excluding the Data view).

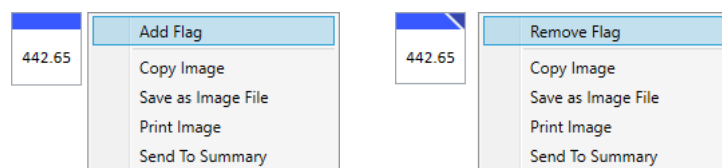


Figure 104. Right-click menu option to add/remove well flags

Use the flag function to highlight specific responses, data points, or entire groups on the plate map for follow-up analysis or investigation. The flagging function is not available in XF Pro Controller software.

When a flag is added (or removed) in one analysis view, all existing analysis views will automatically update the flag status for that well. The flag status will also persist to any new analysis views added to the result file. Flag status will persist from Wave Pro software to Seahorse Analytics and vice versa.

## Bar Graph

The Bar Graph is accessible only on the Overview analysis view, it is located below the Plate Map. The Bar Graph displays the average rate for each group (by default) for a selected measurement. (See [Figure 105](#).) Use the Display drop-down menu to change the rate display from Group (average) to Well (individual well) mode.

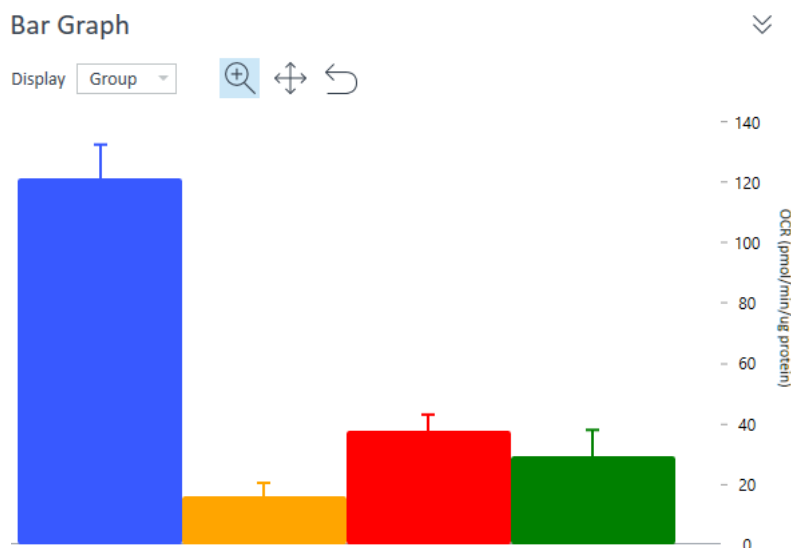


Figure 105. Bar graph

The Bar Chart graph is only available in analysis, not while running an assay. The Bar Chart view provides another way to view individual measurement result data for all groups. The bar chart view is particularly useful for identifying outliers, optimal FCCP concentration, optimal cell seeding density, or other trends that may not be apparent when measurement data is plotted as a kinetic graph.

## Group List (legend)

The Group List is the legend for the data graphed in your analysis view and is found below the graphed data. (See [Figure 56](#).) It provides several functions to modify the data plots:

- Hide groups from the data plot by double-clicking the name of the group. To unhide the data from the graph, double-click the hidden group again. When a group is hidden, the mean and standard deviation of the group will be: Mean: 0.00 and Standard Deviation: 0:00.

## 4 Analyzing Assay Results using Wave Pro

### Group List (legend)

- Display group statistics for the selected rate measurement by checking the **Details** box in the upper-right corner of the Group List. Statistics are displayed as average and error for the selected rate measurement. Select a different rate measurement to display group statistics for that rate. Assay wells that have been turned **OFF** on the Plate Map are not included in the calculated group statistics.

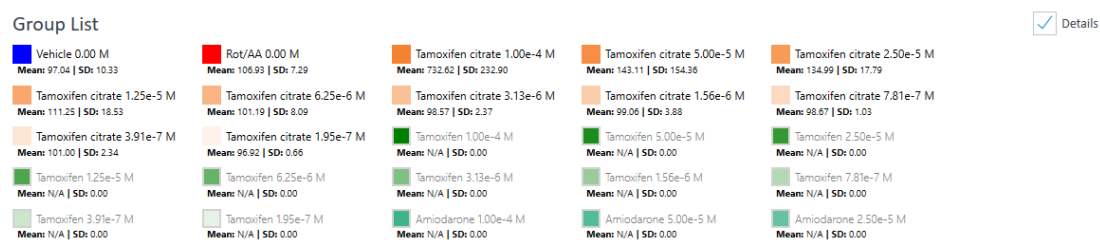


Figure 106. Group list



## Types of Data

### Rate data: OCR, ECAR, and PER

Use the Rate drop-down box to view the rate data options. (See **Figure 107**.) The two rates measured by the Seahorse XF Analyzers during an assay are:

- Oxygen Consumption Rate (OCR); Units: pmol/min
- Extracellular Acidification Rate (ECAR); Units: mpH/min

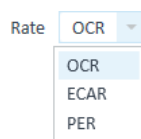


Figure 107. Rate selection drop-down menu

### Level data: O2 and pH

To view Level data for O2 and pH levels for each rate measurement during the assay, use the **Y1**: drop-down. (See **Figure 108**.) The O2 level data is displayed as oxygen tension in units of mmHg. As the biological material in the well consumes oxygen during a measurement, the oxygen tension will decrease.

This decrease in oxygen tension is used to calculate the rate of oxygen consumption (OCR). The pH level data displays the changes in mpH for each rate measurement and is used to calculate ECAR. Level data can be displayed while running an assay, and on the Overview and Data analysis views.

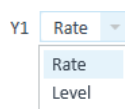


Figure 108. Y1 Drop-down

#### 4 Analyzing Assay Results using Wave Pro

Level data: O2 and pH

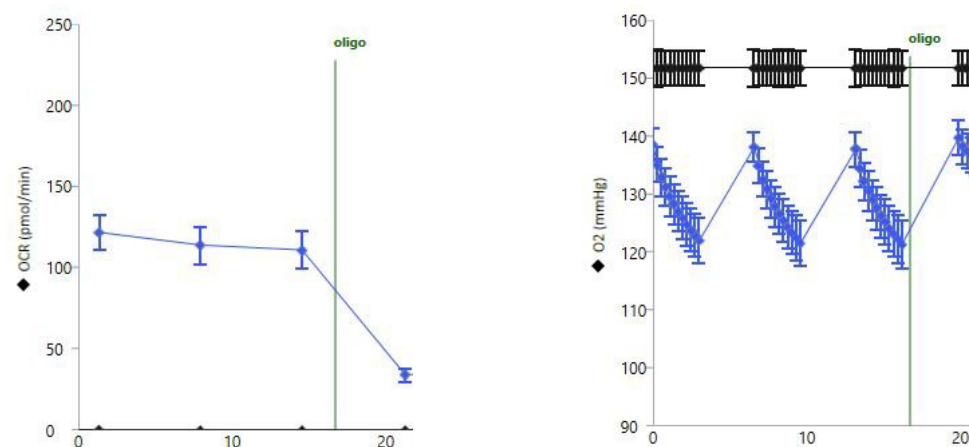


Figure 109. Oxygen Consumption Rate (OCR) data in **Rate** mode (left).  
Oxygen tension (O2) data displayed as mmHg in **Level** mode (right).

## Introduction to Acidification Data

The Seahorse XF Analyzer sensor probes measure changes in the concentration of free protons in the microchamber in real-time. This data is referred to as Extracellular Acidification Rate (ECAR). ECAR can be transformed into a number that reflects the number of protons extruded over time, Proton Efflux Rate (PER). Table 2 shows the types of acidification data.

**Table 2** Types of acidification data

Type of acidification data:	ECAR	PER
Detects acidification from all sources?	Yes	Yes
Changes with media formulation?	Yes	No
Accounts for buffer factor of the system?	No	Yes
How buffering is accounted for?	None	Buffer factor
Displayed while running assay?	No	No

## Proton Efflux Rate

PER is a rate measurement calculated in Wave Pro software. PER delivers a quantitative measure of extracellular acidification and is only available in post-run assay results. It is not displayed while running an assay, ECAR is the only real-time acidification data reported during an XF assay. The three rate options displayed in the **Rate Measurement** drop-down menu are: OCR, ECAR, and PER. PER is calculated using the following equation:

$$\text{PER (pmol H}^+\text{/min)} = \text{ECAR (mpH/min)} \times \text{B.F. (mmol/L/pH)} \times \text{Geometric Volume (}\mu\text{L)} \times \text{Kvol}$$

**Table 3** Wave volume variables

Variable	Unit	Description
Extracellular Acidification Rate (ECAR)	mpH/min	Rate data measured during an assay, reported as the rate of change in mpH in an assay well.
Buffer Factor (BF)	mmol/L/pH	Measure of the in situ buffer capacity obtained in XF instruments, and accounts for both medium buffer capacity and XF assay conditions.
Geometric volume	$\mu\text{L}$	Physical (geometric) volume of the measurement microchamber.
Volume scaling factor (Kvol)	No units	Volume scaling factor used to account for total proton production in the measurement chamber.

Wave Pro software calculates and reports total PER on the Overview, OCR vs. ECAR, and Data analysis views for the XF Pro and XF HS Mini Analyzers. Use the **Rate Measurement** drop-down menu and select **PER** to display on the kinetic graph (Overview analysis view), or scatter plot (OCR vs. ECAR analysis view). To correctly calculate and display PER, the assay media and background well buffer factor must be entered in Wave Pro software.

Wave Pro will automatically display PER if the following conditions are met:

- The assay media assigned in the assay template file is one of the following:
  - Glycolytic Rate Assay Medium (DMEM-based)
  - Glycolytic Rate Assay Medium (RPMI-based)
  - Seahorse XF DMEM Medium, pH 7.4
  - Seahorse XF RPMI Medium, pH 7.4
- The Sensor Cartridge is valid (not expired)
- All background wells have a valid buffer factor value.

The equation displayed above Table 3 shows the PER calculation equation - the only modifiable variable in the equation is Buffer Factor. A default Buffer Factor is automatically used for the PER calculation. If you are using a custom assay medium then Buffer Factor must be determined manually. Refer to the Agilent Seahorse XF Buffer Factor Protocol (available in the XF Glycolytic Rate Assay Report Generator download folder and the XF real-time ATP Rate Assay Report Generator download folder) for guidance.

PER will not be displayed if:

- An assay result file was generated using the spheroid plate type.
- Assay result files that do not have the appropriate Seahorse assay medium assigned to their groups.
- Buffer Factor values are missing from assay medium, group definitions, and background wells.

#### 4 Analyzing Assay Results using Wave Pro

If PER is not displayed:

### If PER is not displayed:

- 1 Open the assay result file in Wave Pro software
- 2 Click **Modify**.
- 3 Click **Add** next to **Assay Media** (See [Figure 110](#))



Figure 110. Assay Media option

- 4 To select the assay medium, use the **Media** catalog drop-down menu. (See [Figure 111](#).)
  - Seahorse XF DMEM Medium, pH 7.4
  - Seahorse XF RPMI Medium, pH 7.4
  - Glycolytic Rate Assay Medium (DMEM-based)
  - Glycolytic Rate Assay Medium (RPMI-based)

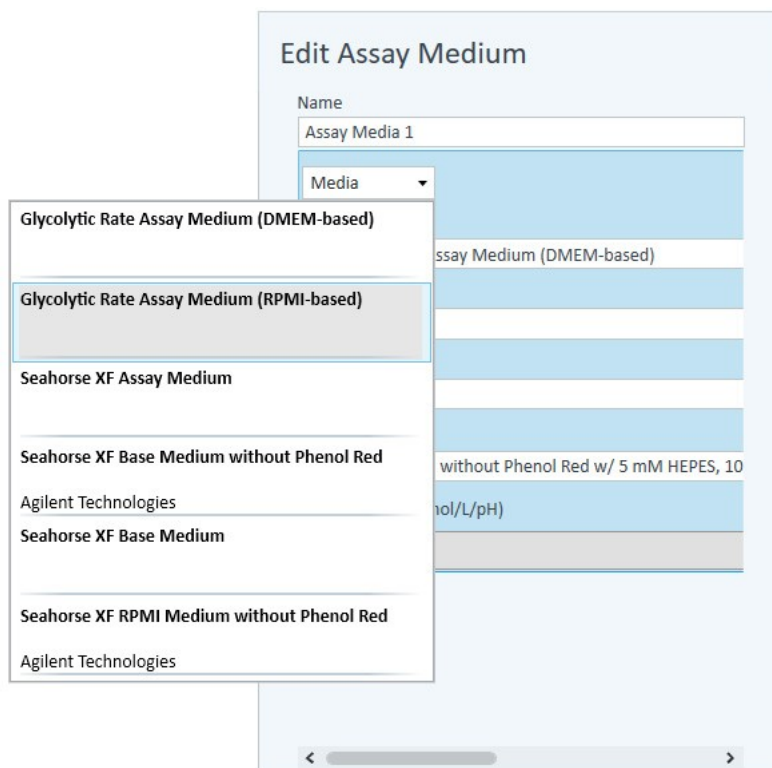


Figure 111. Assay medium media menu

- 5 To configure Background Well Buffer Factor, click **Assay Media**.
- 6 Click **Configure**.

#### 4 Analyzing Assay Results using Wave Pro

If PER is not displayed:

- 7 Under the column header **Default Buffer Factor**, check the boxes next to each background well, and click **Save** (See [Figure 112](#)).

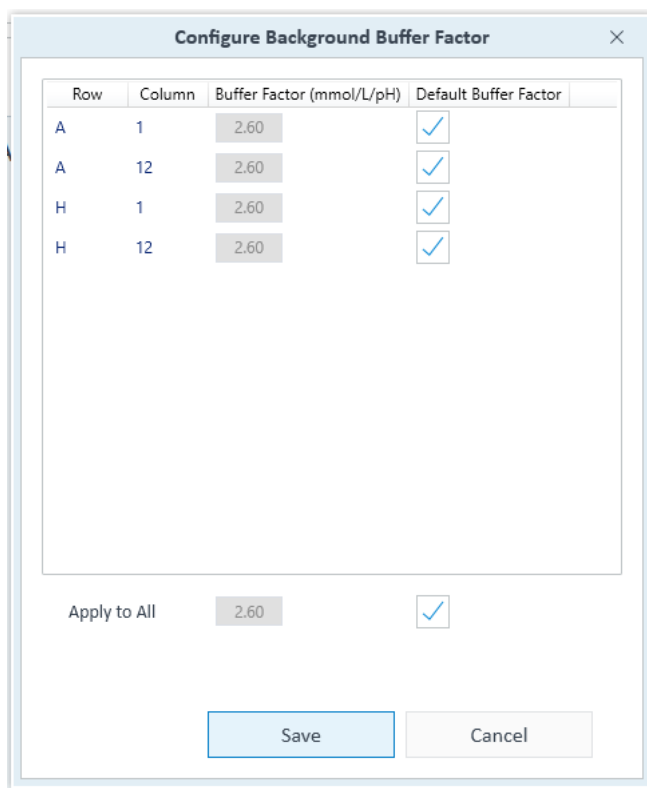


Figure 112. Configure background buffer factor

If the background well buffer factor values have not been configured, Wave will show an "attention" icon next to the rate measurement drop-down menu. (See [Figure 113](#).)

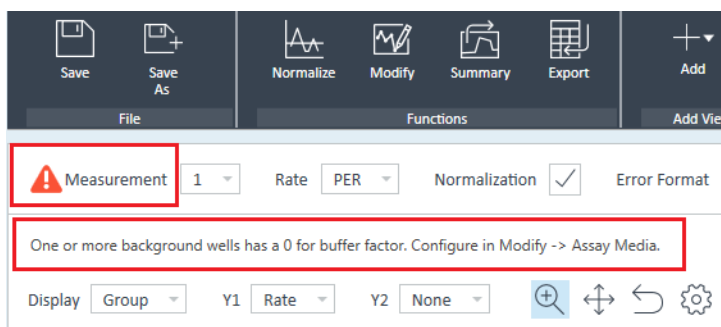


Figure 113. Alert when background well buffer factor is not configured

#### NOTE

When editing your assay template file follow steps 5-7 above to configure the background well buffer factor before running an XF assay.

- 8 Assign the **Assay Media** to each group in the group list. First click **Collapse/Expand All** to display the Group Definitions for each group.

## 4 Analyzing Assay Results using Wave Pro

If PER is not displayed:

- 9 Use the **Media** drop-down menu to add the assay media to each group (Seahorse XF DMEM or RPMI Medium, pH 7.4; or Glycolytic Rate Assay Medium (DMEM-based or RPMI-based)).

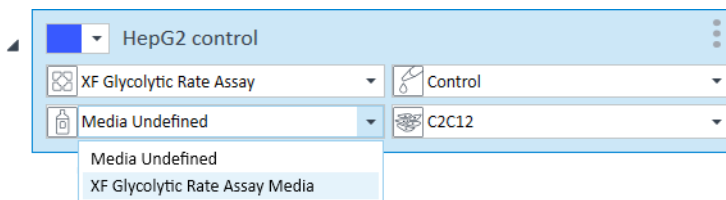


Figure 114. Media drop-down menu

- 10 When finished, click **Apply**.
- 11 The kinetic graph and scatter plot will display PER data when selected using the **Rate** drop-down menu.
- 12 When finished with analysis, save the changes made to the result file.

### Display PER for assays run with custom assay media

Prior to running the assay, calculate the Buffer Factor of the custom assay medium. Follow the steps in the *Agilent Seahorse XF Buffer Factor Protocol* (available in the XF Glycolytic Rate Assay Report Generator download folder and the XF Real-Time ATP Rate Assay Report Generator download folder) for guidance.

- 1 Open the assay result file in **Wave Pro** software.
- 2 Click **Modify**.
- 3 Click **Add** next to **Assay Media**.
- 4 In the Edit Assay Medium window, enter the **Buffer Factor** in the field provided.
- 5 Next, click **Assay Media** again to configure Background Well Buffer Factor.
- 6 Click **Configure**.

#### 4 Analyzing Assay Results using Wave Pro

Export total PER data

- Under **Buffer Factor (mmol/pH)**, type in the same Buffer Factor value and click **Save**. (See [Figure 115](#).)

Row	Column	Buffer Factor (mmol/L/pH)	Default Buffer Factor
A	1	2.40	<input type="checkbox"/>
A	12	2.40	<input type="checkbox"/>
H	1	2.40	<input type="checkbox"/>
H	12	2.40	<input type="checkbox"/>

Apply to All:  ☐

Figure 115. Configure background buffer factor

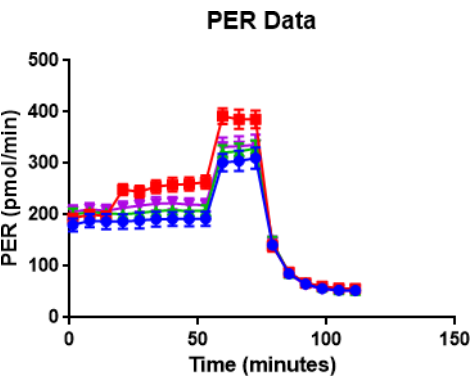
- Assign the **Assay Media** to each group in the group list. First click **Collapse/Expand All** to display the Group Definitions for each group.
- To select the appropriate medium for each group, use the **Media** drop-down menu.
- When finished, click **Apply**.
- The kinetic graph and scatter plot will display PER data when selected using the Rate drop-down menu.
- When finished with analysis, save the changes made to the result file.

## Export total PER data

PER data is exported to both Microsoft Excel and GraphPad Prism file exports. (See [Figure 116](#).) For manual PER data analysis or graphing purposes, export data from Wave Pro to either file type using the **Export** function located in the top-level ribbon.



4 Analyzing Assay Results using Wave Pro  
Export total PER data



GraphPad Prism

PER (pmol/min)				
Time				
	A02	A03	B02	B03
1.30	160.21	167.88	180.45	171.75
7.75	168.53	176.70	188.61	173.25
14.21	166.19	171.25	191.04	167.33
20.74	166.05	169.32	188.96	169.84
27.19	168.23	173.25	187.72	169.88
33.63	172.70	175.33	190.88	172.73
40.08	173.67	175.65	193.54	176.15
46.52	170.06	178.75	196.05	176.07
52.97	176.57	177.38	194.53	175.99
59.50	273.48	287.51	311.39	283.60
65.95	276.34	288.50	309.63	282.39
72.39	286.07	292.71	317.15	285.45
78.93	133.56	141.12	138.86	136.23
85.38	82.85	86.43	81.25	80.42
91.85	62.59	65.72	60.55	60.62
98.30	57.22	58.31	51.40	54.00
104.76	54.86	53.36	50.32	48.28
111.21	54.26	54.01	47.10	48.72

Microsoft Excel

Figure 116. Prism data export examples

## Baseline to a Rate Measurement (%)

You may choose to display rate data as a percent of (1) a selected rate measurement or (2) a percent of a control group in Wave Pro. The Baseline function available on the Overview and OCR vs. ECAR analysis views provide this functionality. To express kinetic data as a percent of a selected rate measurement for all groups, use the **Baseline** drop-down menu and select a **Rate** measurement. (See [Figure 117](#).)

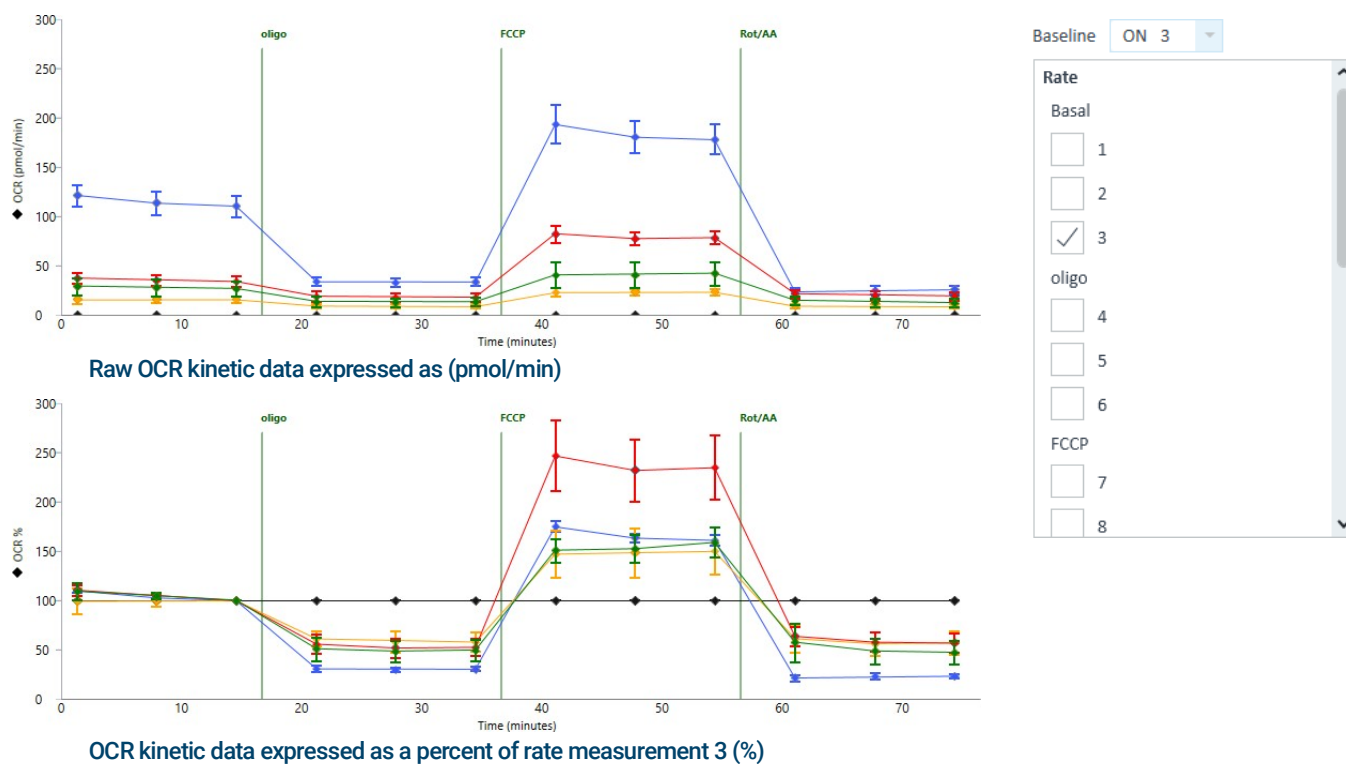


Figure 117. Baseline to a rate measurement

## Baseline to a Control Group (%)

To display rate data as a percent of a Control Group, using the same baseline drop-down menu, scroll down to display and select your Control group. The example in **Figure 118** shows how this feature can provide a better graphical representation of the effect of glucose inhibition on OCR and facilitate data interpretation.

A Control group (blue line) with only media injections is selected as the baseline control group. After selection, each rate measurement in the groups "Glucose Dependency" (orange line) and "Glucose Capacity" (green line) is expressed as a percentage of the rate measurement of the Control group.

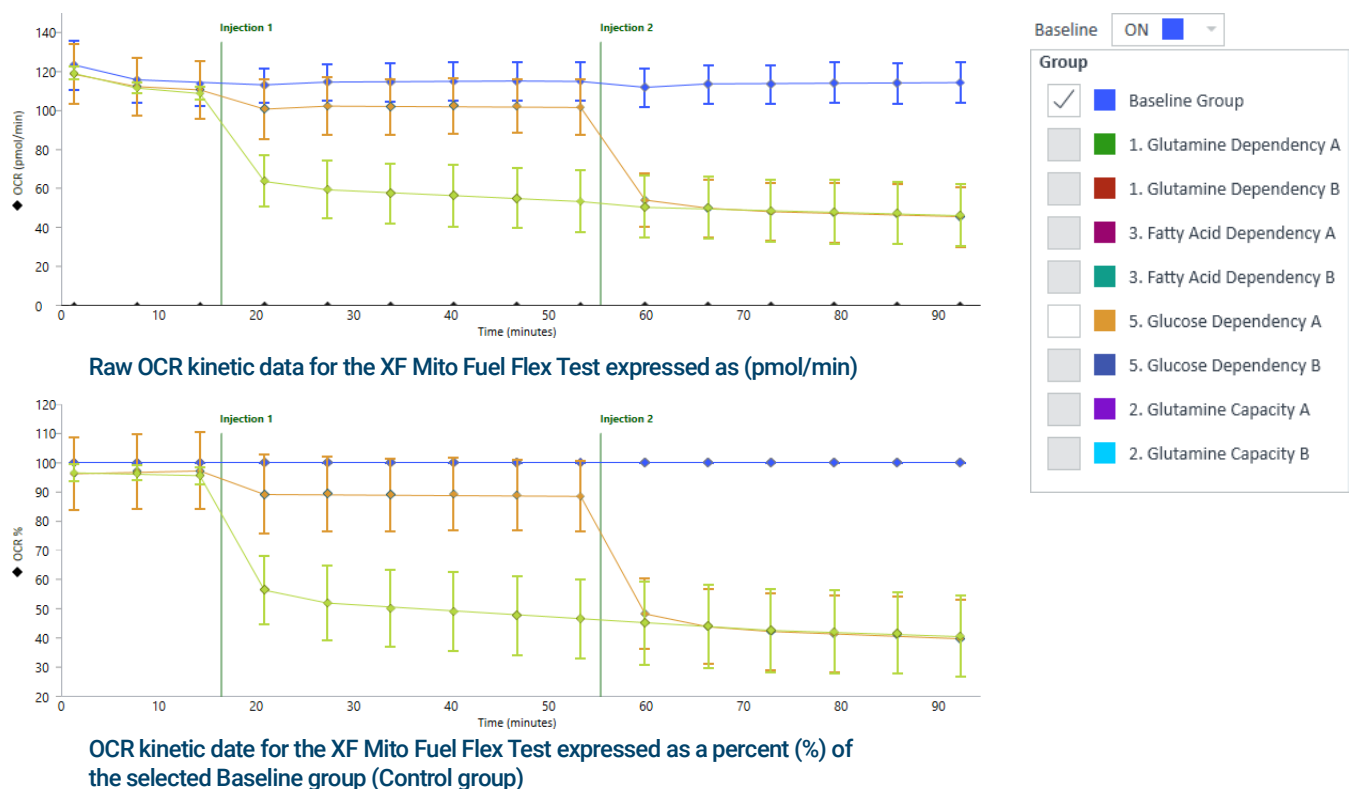


Figure 118. Baseline to Control group

## Standard Deviation and Standard Error of the Mean

Two types of error bars in Wave Pro and XF Pro software are Standard Deviation (**StdDev**) and Standard Error of the Mean (**SEM**). Use the **Error Format** drop-down menu to change the error bar type displayed on the kinetic graph/scatter plot and the Group List. (See Figure 73.) to hide error bars from the data graph, select **None**. In **Well** mode, error bars are not displayed.

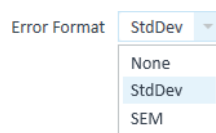


Figure 119. Error Format menu

## Rate Overlay: OCR, ECAR, PER, O2, or pH

In Wave Pro (only), compare two sets of data (rate and/or level) on one kinetic graph using the **Y2** drop-down menu. This will overlay the Y2 data with the selected Y1 data. (See **Figure 120**.)

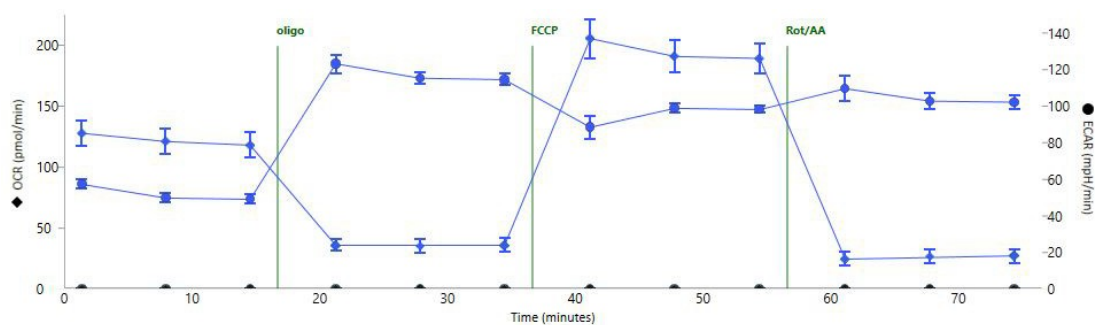


Figure 120. Y2 rate overlay

## Customizing Data Displays

### Excluding assay wells

When an assay result file is opened for the first time, data from all assay wells are displayed on the kinetic graph(s) & included in group calculations. To remove an assay well in the graphed data and calculated group statistics for that group, simply click the well on the Plate Map. Assay wells that are turned OFF have a grey background on the Plate Map. Assay wells that are included in graphed data and calculated group statistics have a white background. (See **Figure 121**.) Assay wells must be manually turned off from each analysis view. Turning off an assay well on one analysis view is not automatically applied to other open (or new) analysis views.

12.14	17.82
13.35	11.19

The upper 2 assay wells in this group are turned OFF and excluded from the graphed data and group statistics.

12.14	17.82
13.35	11.19

All assay wells in this group are included in the graphed data and group statistics.

Figure 121. Two states of assay wells

Column and row headers can be used to exclude entire vertical or horizontal sections of a Plate Map. Click the numerical column header or alphabetical row header to turn the assay wells in that column or row **OFF**.

Click the small triangle in the upper-left corner of the Plate Map to turn off all assay wells from the graphed data. (See **Figure 122**.)

**Plate Map**

OCR (pmol/min)

	1	2	3	4
A	0.00	117.26	83.94	30.82
B	123.80	148.13	123.36	43.32
C	123.66	133.67	149.37	42.06
D	125.15	125.59	141.14	28.33

Figure 122. Corner toggle to turn OFF all wells on the Plate Map

## Display modes: group and well

Rate data is graphically displayed in Group mode on all analysis views by default, displaying average and error of the wells assigned to the group. Switch to **Well** mode to display individual kinetic traces (or data points for OCR vs. ECAR view) for each assay well assigned to the group. Error bars are not displayed in Well mode.

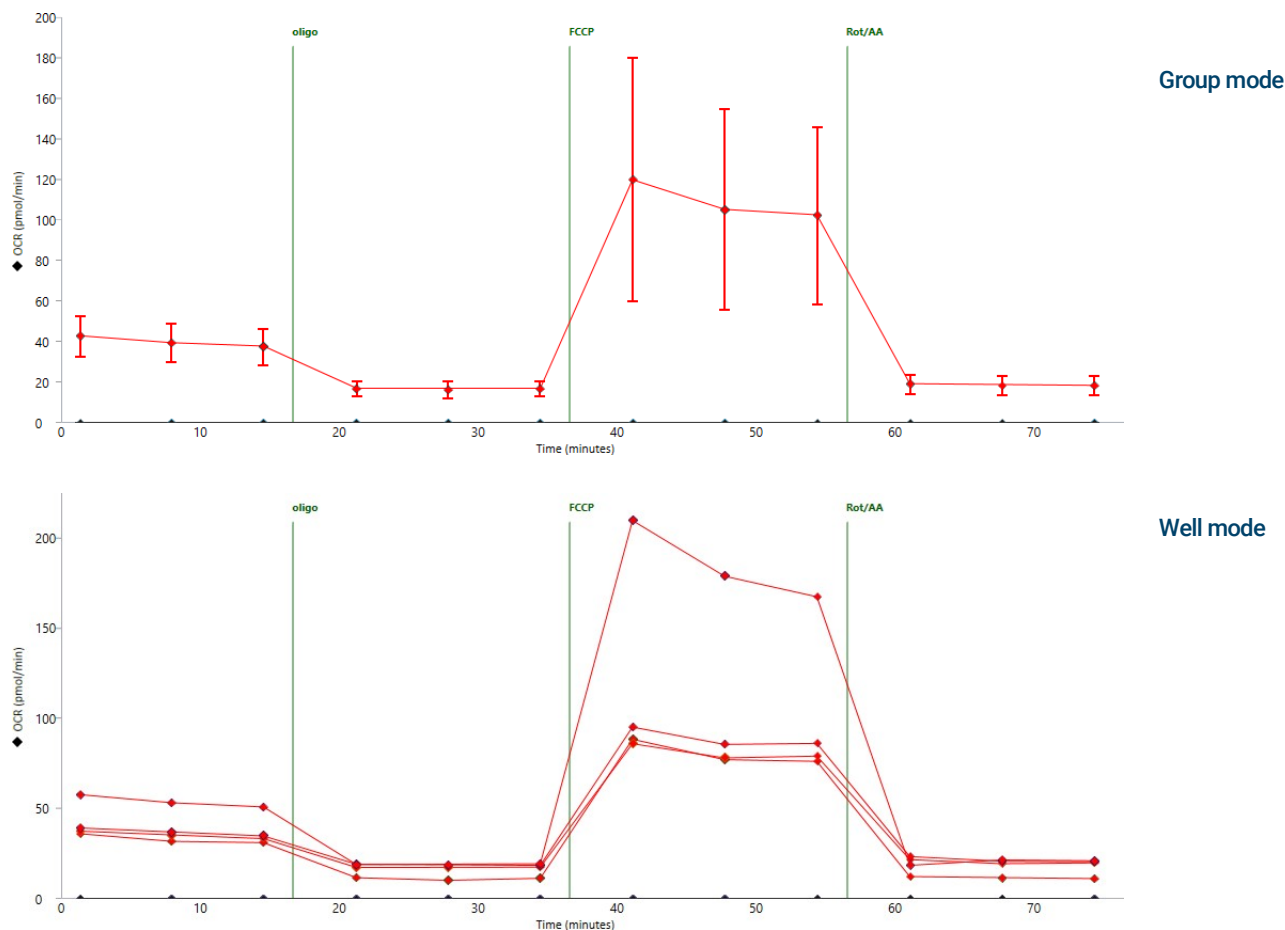


Figure 123. Example of an OCR kinetic graph in Group (average) and Well mode

### Resize and Adjust view areas

View areas on the different analysis views can be adjusted in two ways: using the gray borders (red arrows in [Figure 124](#)) and the << and >> arrows (outlined in red in [Figure 124](#)) to hide entire regions of the view (Overview pictured in [Figure 124](#)). Use the border between the kinetic graph and Plate Map to elongate/shrink the graph or Plate Map size. Hide the Group List to increase the vertical size of the kinetic graph using the border between the kinetic graph and Group List. Hide the Bar Chart using the border between the Plate Map and Bar Chart.

## 4 Analyzing Assay Results using Wave Pro

### Graph display options

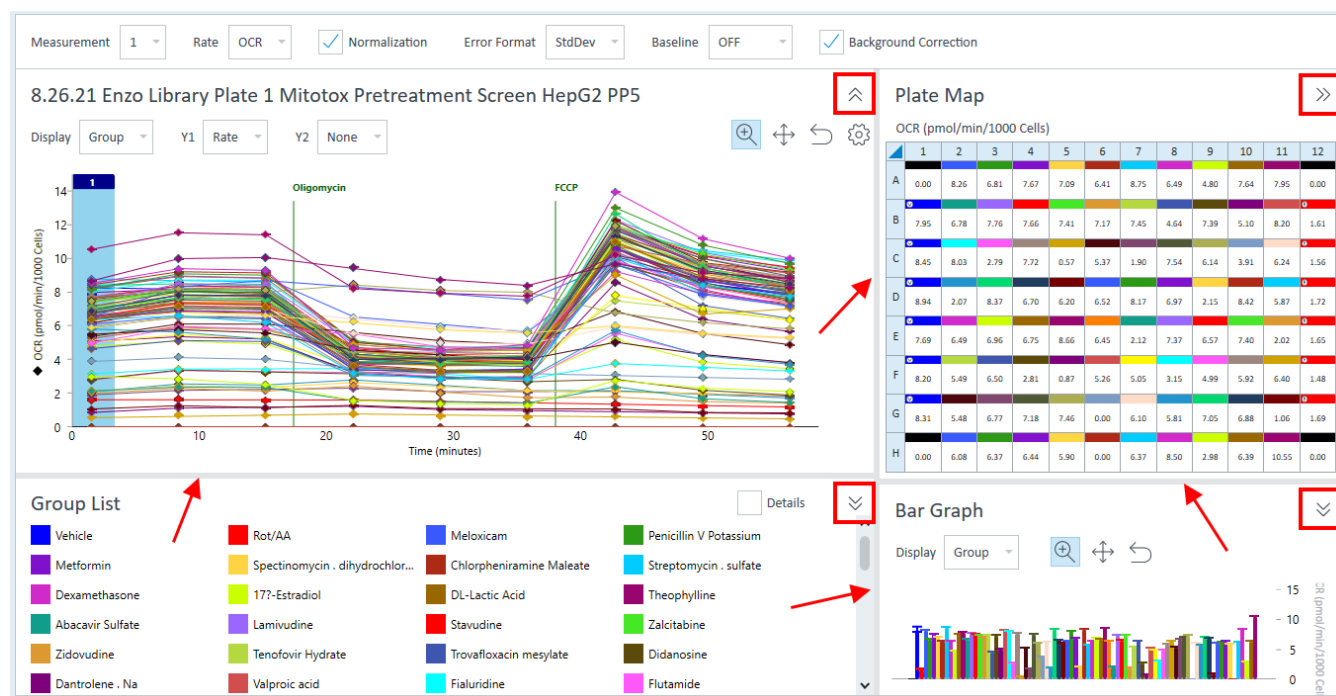


Figure 124. View areas

### Zoom, Pan, and Restore

The Zoom, Pan, and Restore buttons are displayed above the kinetic graph on the Overview and the scatter plot on the OCR vs. ECAR view.



**Zoom** Right-click and hold to select an area on the kinetic graph or scatter plot to magnify the selected area.



**Pan** After zooming into a section of a graph, use the **Pan** button to move around the graph while keeping the selected zoomed display aspect ratio.



**Restore** Return the graph to full view.

### Graph display options

To show the graph display formatting options menu, click the cog next to the restore button.



## 4 Analyzing Assay Results using Wave Pro

### Graph display options



Kinetic Line Chart Options

Graph Title: 8.26.21 Enzo Library Plate 1 Mitotox Pretreatment Screen HepG2 PP5

Y1 Axis		Y2 Axis		Time Axis	
Min	Max	Min	Max	Min	Max
▲	▲	▲	▲	▲	▲
0.00	15.00	0.00	0.00	0.00	58.73
▼	▼	▼	▼	▼	▼
<input checked="" type="checkbox"/> Auto Scale		<input checked="" type="checkbox"/> Auto Scale			
Interval	Thickness	Interval	Thickness	Interval	Thickness
▲	▲	▲	▲	▲	▲
	0.00		0.00		0.00
▼	▼	▼	▼	▼	▼
<input checked="" type="checkbox"/> Show Error Bars		<input checked="" type="checkbox"/> Show Error Bars		<input checked="" type="checkbox"/> Show Rate Highlight	
<input checked="" type="checkbox"/> Show Zero Line		<input type="checkbox"/> Show Zero Line		<input checked="" type="checkbox"/> Show Injection Markers	
<input checked="" type="checkbox"/> Line Markers		<input checked="" type="checkbox"/> Line Markers			
<input type="checkbox"/> Point to Point		<input type="checkbox"/> Point to Point			

Figure 125. Example of the kinetic graph display settings

- **Minimum** and **Maximum**: Set the minimum and maximum bounds for the Y1, Y2, and X-axes.
- **Interval**: Set the major units for the Y1, Y2, and X-axes.
- **Thickness**: Add and adjust thickness of Y1, Y2, and X-axes gridlines. Adjust values in 0.50 intervals. Default value is '0.00' (no gridlines displayed).
- **Show Error Bars**: Toggle error bars **ON/OFF**.
- **Show Zero Line**: Display a horizontal reference line at the zero when the y-axis is negative.
- **Point-to-Point**: Display point-to-point rates for the selected axis.
- **Show Rate Highlight**: Toggle **ON/OFF** display of the vertical blue rate highlight bar on the kinetic graph (indicates the selected rate measurement that corresponds to values displayed on Plate Map).
- **Show Injections Marker**: Toggle the vertical injection lines on the kinetic graph **ON/OFF**.
- **Heat Map Color Options**: Adjust Color Tolerance and assign color to High and Low Value of the heat map.

## 4 Analyzing Assay Results using Wave Pro

### Graph display options

- **Heat Map Graph Options:** Set the Color Tolerance from the drop down in the Hat Map settings. Lower tolerance percentage will accentuate value transition thresholds. You can also select the colors used for High and Low values

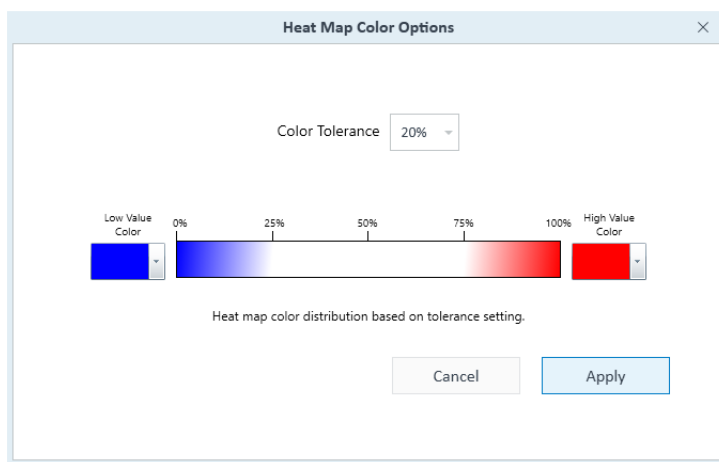


Figure 126. Heat Map color options

- **Dose Response Graph options:** Adjust the graph title, y-axis min, max, interval values, line thickness value, as well as the dose axis scale setting (logarithmic or linear) and the min, max and line thickness values.

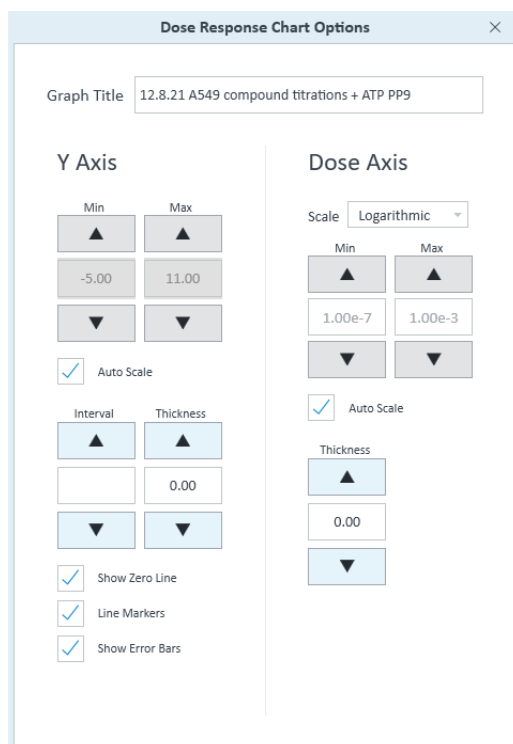
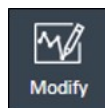


Figure 127. Dose Response Chart options

## Modifying Assay Result Files

Click **Modify** (in the **Functions** ribbon) to add or edit details of an assay result file, for example to modify a group name, edit buffer factor values, or to unassign a well from a group calculation. The Group Definitions view is always displayed first when opening Modify mode.



The views in Modify mode look nearly identical to the template designer views. When actively modifying an assay result file, Wave Pro will indicate the program is in Modify mode in the upper-left corner of Wave. To return to assay result data, click **Apply** (upper-right corner) to apply modifications to the result file, or click **Cancel** to cancel out of Modify mode and prevent any changes from taking effect.

Modify Assay Mode

Apply

Cancel

### NOTE

The **Modify** function does not enable editing result data acquired during the assay.

## Group definitions

To display the Injection Strategies, Pretreatments, Assay Media and Cell Type details entered in the assay template prior to running the assay, select Group Definitions. Modifications to the **Group Definitions** tab include:

- Renaming assay groups
- Reconfigure details for one or more groups
- Add additional group information that was left out of the initial assay template design, or
- Add Buffer Factor to calculate PER

## Plate Map

To display and edit conditions of each assay group using the drop-down menus below the group name, select **Plate Map**. This tab also enables reassigning group locations on the Plate Map. Select a group, then select the appropriate assay wells on the Plate Map.

## Injection Names

To edit the name of each compound injection during the assay, select **Injection Names**. The specified injection names from the Instrument Protocol tab are displayed above each injection line on a kinetic graph. The default name of each injection is: 'Injection 1', 'Injection 2', 'Injection 3', and 'Injection 4'. Type an Injection Name for each compound injection, and click **Apply** to display the new name above the corresponding injection line.

## General Information

To edit details of an assay result file, such as: Project Name, PI, or to add / edit notes about the data file, select **General Information**.

## Normalization

In the context of an XF assay, normalization is the process of recalculating rate data to account for variations in a relevant cell parameter (such as cell count or protein concentration) that can cause well-to-well variability in XF data. The Normalization function provides a simple mechanism to apply normalization data to your XF rate data. To use the normalization function, an independent assessment of the assay wells for cell number, DNA content, protein concentration or similar parameter is required. (See **Figure 128** on page 112.)



To normalize data in Wave Pro, you must supply:

- **Normalization data (required):** The numeric data generated from the independent assessment of the well (cell count, protein concentration, DNA content, and so forth).
- **Normalization unit (required):** This alphanumeric field describes the units to which the data are to be normalized. It comprises the desired value to which the data will be normalized (see Normalization Scale Factor) plus the unit of measure of your normalization values (such as "cells", "mg", "ng", and so forth).
- **Normalization Scale Factor:** This number determines what value the rate data will be normalized to. Default is 1 and adjustment is optional.

### Edit Normalization Mode

Normalization Unit

Scale Factor

µg protein content

1

Normalization Values

	1	2	3	4	5	6	7	8	9	10	11	12
A	.218	1.786	1.918	2.226	1.986	2.205	1	1.048	1.072	1.402	1.038	.251
B	2.133	1.697	2.069	1.633	1.654	1.949	1.11	1.05	1.071	1.19	1.014	.962
C	1.792	1.945	1.772	1.916	1.777	1.925	1.06	1.122	1.104	1.208	1.108	1.029
D	1.998	1.988	1.597	2.084	1.732	1.696	1.047	1.256	1.04	1.097	1.065	.995
E	1.839	1.816	1.944	1.664	1.858	1.931	1.116	1.026	1.052	1.035	1.173	.979
F	1.943	1.631	1.796	1.696	1.808	1.975	1.008	1.058	1.058	1.158	1.042	1.06
G	1.983	1.753	1.876	1.749	1.727	1.637	1.132	1.077	1.027	1.007	1.038	1.068
H	.407	1.744	1.758	1.784	1.681	1.728	1.015	1.032	1.028	.995	1.081	.241

Select All

Paste

Figure 128. Edit & apply normalization data

## Add normalization data

Click **Normalize** (in the **Functions** ribbon) to display the Edit Normalization Mode. The Normalization Values table is the place to copy/paste normalization data. The simplest method of adding normalization data is to copy / paste:

- 1 Open your file containing normalization data. Normalization data must be in the same plate map layout as the Normalization Values table.
- 2 Select the normalization data and copy to the clipboard **[Ctrl+C]**.
- 3 On the normalization view, click **Select All > Paste**.
- 4 Click **Apply**.

## 4 Analyzing Assay Results using Wave Pro

### Add normalization data

You can also manually type each normalization data point into the Normalization table, however this can be time consuming.

#### NOTE

**Background wells should be left blank on the Normalization Table.**

#### Normalization unit

Before you can apply normalization values to rate data, a Normalization Unit is required. The Normalization Unit is displayed on the Y1 axis title on the kinetic graph when normalization is applied and checked **ON**.

##### Normalization Unit

µg protein

For example, adding µg Protein to the **Normalization Unit** field will update rate displayed for Y1 axis label to: (pmol/min/µg Protein). If you have added a Normalization Scale Factor as well, be sure to add the scale factor value to the Normalization Unit field.

#### Normalization scale factor

The Normalization Scale Factor allows you to scale normalized data to a specific factor, such as cell number. It is often preferable to normalize to an average or rounded number.

##### Scale Factor

1

Enter a scale factor value to recalculate and multiply normalized rate data based on the equation shown below:

Normalized Rate (well)=[Rate (well)/(Normalization Value)]\*Normalization Scale Factor

For example, if assay wells contain values of 12052, 12503, and 12757 cells per well, a user might enter 12000 as the normalization scale factor.

#### NOTE

**Be sure to enter the same value in the Normalization Unit field to display the factor correctly on the y-axis. For example, if using cell count and the scale factor is 10000, enter 10000 cells as the Normalization Unit. After clicking Apply, the Y1 axis will display OCR as (pmol/min/10000 cells) rather than OCR (pmol/min/cells)**

## 4 Analyzing Assay Results using Wave Pro

### Add normalization data

#### Viewing normalized data

The **Normalization** checkbox will become active on all analysis views after applying normalization data to your assay result file. Use the checkbox to quickly toggle the rate data display between non-normalized rates and normalized rates on the kinetic graph, scatter plot, and Plate Map. All data graphs will display the Normalization Unit entered. (See [Figure 129](#).)

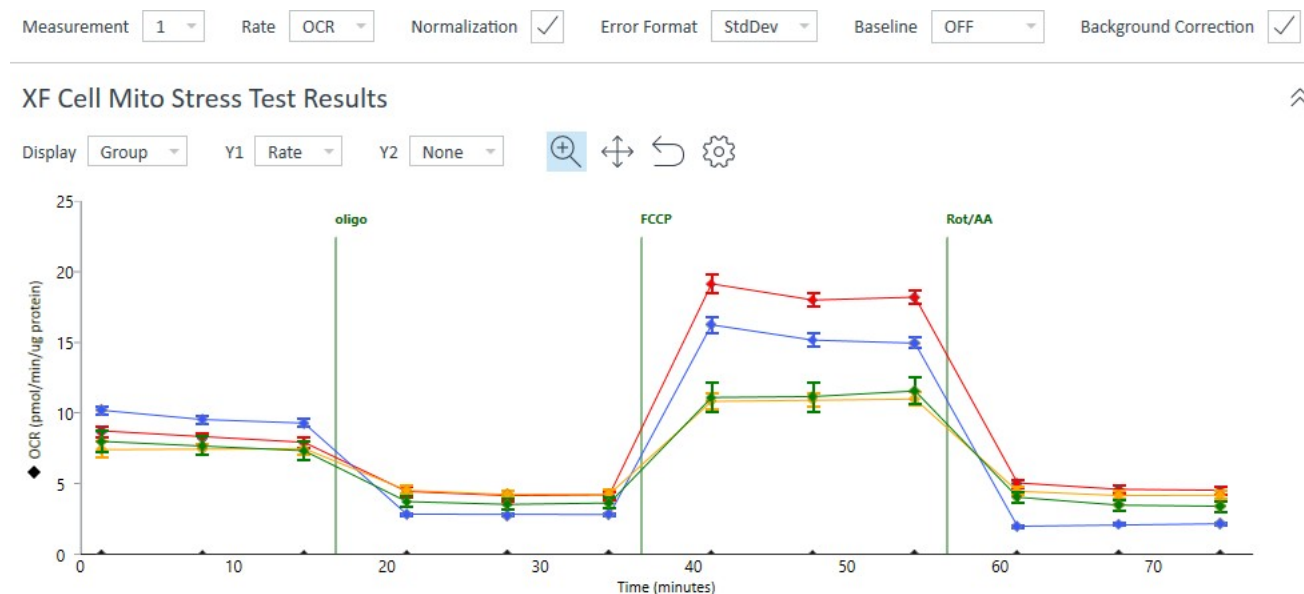


Figure 129. Normalized rate data in a kinetic graph

## Data Export Options

Export assay result data for additional analysis, graphing, or other purposes. Use the **Export** button (from any analysis view) to display default export options. The most comprehensive data exports are the **Microsoft Excel** and **GraphPad Prism** options. You can also export result data to an Agilent Seahorse XF Report Generator. XF Report Generators provide standardized analysis templates for each Seahorse XF Assay Kit, however if you choose to perform this type of analysis, it is strongly encouraged using **Agilent Seahorse Analytics** over XF Report Generators.



### Export to Microsoft Excel

To export assay result data to Microsoft Excel:

- 1 Click **Export** (in the Functions ribbon).
- 2 Click **Microsoft Excel**.
- 3 Choose a save location and click **Save**.

Using the export function described above, there will be between 7-13 tabs in the exported Excel file depending on how you configured your result file.

**Raw data:** Exporting raw result data (not normalized, baseline OFF) will have seven data tabs. (See **Figure 130**.)

Measurement 1		
	1	
A	0.00	
B	46.24	
C	36.60	
D	44.44	
E	64.52	
F	56.29	
G	47.87	
H	0.00	

Rate (Plates) Tab: Raw OCR rates for rate measurement 1 (from an 8-well XF HS Mini Analyzer).

Figure 130. Raw rate data

- a Assay configuration:** Information from the assay result file including the Group Layout (Plate Map), Cartridge/Cell Plate Barcode and other information, Instrument Protocol, and normalization data and normalization unit.
- b Rate:** Raw OCR, ECAR, and PER data in a column format organized by measurement.
- c Rate (Columns):** Raw OCR, ECAR, and PER data in a column format organized by group. Similar output format to the GraphPad Prism export.
- d Rate (Plates):** Raw OCR, ECAR, and PER data organized by measurement displayed as a Plate Map.
- e Raw:** Raw assay well data for temperature, emission values and reference values. Often used for troubleshooting by **Agilent Cell Analysis Technical Support**.
- f Calibration:** Raw calibration data for both the O2 and pH channels.
- g Operation log:** List of each protocol command executed by the analyzer, timing of the command, and outcome (success/fail).

## 4 Analyzing Assay Results using Wave Pro

### Export to Microsoft Excel

**Normalized data:** Exporting result data that has been normalized (baseline OFF) will have three additional data tabs in the Excel export. (See [Figure 131](#).)

Measurement 1	
	1
A	0.00
B	0.00385
C	0.00305
D	0.00370
E	0.00538
F	0.00469
G	0.00399
H	0.00

Normalized Rate (Plates) Tab:  
Normalized OCR rates for rate measurement 1 (from an 8-well XF HS Mini Analyzer).

Figure 131. Normalized rate data

- a Normalized rate:** Normalized OCR, ECAR, and PER data in a column format organized by measurement.
- b Normalized rate (Columns):** Normalized OCR, ECAR, and PER data in a column format organized by group. Similar output format to the GraphPad Prism export.
- c Normalized rate (Plates):** Normalized OCR, ECAR, and PER data organized by rate, displayed as a Plate Map.

**Baselined data:** Exporting result data with baseline ON (recalculated as a percent of a rate measurement or of a control group) will have three additional data tabs in the Excel export. (See [Figure 132](#).)

Measurement 1	
	1
A	100.00
B	128.34
C	139.25
D	124.75
E	126.51
F	124.18
G	128.18
H	100.00

Baselined Rate (Plates) Tab:  
Baseline OCR rates for rate measurement 1 displayed as a percentage (from an 8-well XF HS Mini Analyzer).

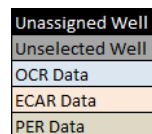
Figure 132. Baseline rate data

- a Baselined rate:** Baselined (%) OCR, ECAR, and PER data in a column format organized by measurement.
- b Baselined rate (Columns):** Baselined (%) OCR, ECAR, and PER data in a column format organized by group. Similar output format to the GraphPad Prism export.
- c Baselined rate (Plates):** Baselined (%) OCR, ECAR, and PER data organized by rate, displayed as a Plate Map.



### Excel export legend

The legend on each tab indicates the assay wells that have been excluded from calculations (turned **OFF** or unassigned) in Wave Pro prior to export. (See [Figure 133](#).) Assay well B in the image below was turned **OFF** in Wave Pro before exporting to Excel.



Unassigned Well
Unselected Well
OCR Data
ECAR Data
PER Data

Figure 133. Plate map legend in Excel export.

### Export select graph data to Microsoft Excel (from any analysis view)

To export customized analysis view data from Wave Pro to Microsoft Excel, you can right-click on a graph (or plate map) to display the Excel export option.

- 1 Right-click anywhere on a chart or **Plate Map** to display an option menu and select **Export Graph Data to Excel**. (See [Figure 134](#).)

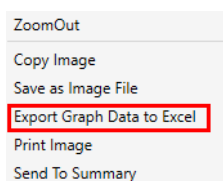


Figure 134. Option menu

- 2 Browse to the desired save location, type in the file name for the export.
- 3 Click **Save**.

The data values exported to Microsoft Excel will be in the exact same format as they are displayed on the chart or Plate Map.

#### NOTE

**This Microsoft Excel file export is not compatible with the XF Report Generators. Use the Export button to export data directly to the XF Report Generators from any analysis view.**

## Export to GraphPad Prism

To export assay result data to GraphPad Prism:

- 1 Click **Export** (on any analysis view).
- 2 Select **GraphPad Prism**.
- 3 Choose a save location and click **Save**.

#### NOTE

**Prism export compatibility has been validated using GraphPad Prism 7 and later.**

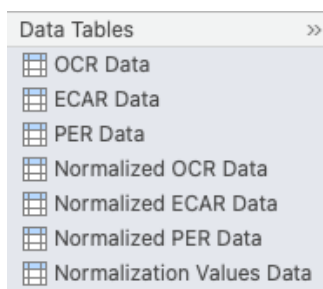
### GraphPad Data tables

Using the Prism export function, Wave Pro will export rate data (OCR, ECAR, PER) as:

## 4 Analyzing Assay Results using Wave Pro

### Export to GraphPad Prism

- **Raw rate data:** always exported
- **Normalized rate data:** Normalization data must be applied to the assay result file in Wave before export
- **Baseline rate data:** Rate data as a percent of the selected baseline measurement or control group



Sample data output in GraphPad Prism from an 8-well XF HS Mini Analyzer with two assay groups, **Disease Group** and **Healthy Group**. (See [Figure 135](#).)

X	Group A			Group B		
Time (minutes)	Disease Group			Healthy Group		
X	A:Y1	A:Y2	A:Y3	B:Y1	B:Y2	B:Y3
1.5591	36.5971	44.4419		64.5248	56.2883	47.8664
8.0789	30.7173	39.6487		56.5285	49.8633	41.6654
14.6040	27.7426	36.9345		53.6014	46.7723	38.8526
21.1255	26.2812	35.6242		51.0053	45.3292	37.3431
27.7413	14.0176	17.1571		25.6648	22.5966	17.3317
34.2693	13.7931	17.2244		26.3632	23.8603	18.1830
40.7907	13.7985	16.4647		25.5408	23.3045	17.3062
47.3159	13.1798	15.6891		24.9620	22.5543	17.4538
53.9360	37.7011	29.9699		74.9459	69.0456	53.5484
60.4695	32.8831	24.3168		69.2413	61.1567	50.3059
66.9959	30.8536	20.7413		63.5366	56.0195	46.1478
73.5296	28.8017	17.9396		59.6601	52.3409	43.6067
80.1601	7.5714	8.5284		14.9611	13.7690	8.6728
86.6962	7.5500	7.7343		15.7009	14.1531	9.1453
93.2338	7.0782	8.9587		14.9156	14.4237	8.4967
99.7749	5.9633	6.9644		14.0657	12.4098	7.0267

Figure 135. Prism data output

- **Column-format Data Display:** Time is a fixed column next to the assay groups, and will scroll horizontally when there are more assay groups.
- **Assay Wells and Columns:** The number of columns with rate data (below each group header) reflects the number of assay wells in each group.
- **Excluded Assay Wells:** Assay wells that have been excluded in Wave Pro (excluded or unassigned) will appear as an empty column in Prism. Column A:Y3 below Group A (called Disease Group) in [Figure 135](#) on page 118 shows rate data that has been exported for two wells in the Disease Group. Refer to Project Info tab in Prism, or the assay result file in Wave Pro, for excluded assay wells in the exported Prism file.
- **Rows and Rate Measurements:** The number of rows reflects the number of rate measurements captured during the assay. In [Figure 135](#) on page 118, there are a total of 16 measurements performed during the assay (16 rows).

GraphPad Kinetic graphs

Every Data Table in the GraphPad Prism file export also contains a corresponding kinetic graph. The graph in **Figure 136** represents the data set (previous page) exported from the XF HS Mini Analyzer. The Graphs folder displays the available kinetic graphs based on the type of data exported from Wave Pro, that is, raw data, normalized data, or data as a percent of the baseline. Assay wells or groups that are turned OFF in Wave Pro prior to export will be excluded from the Prism file export.

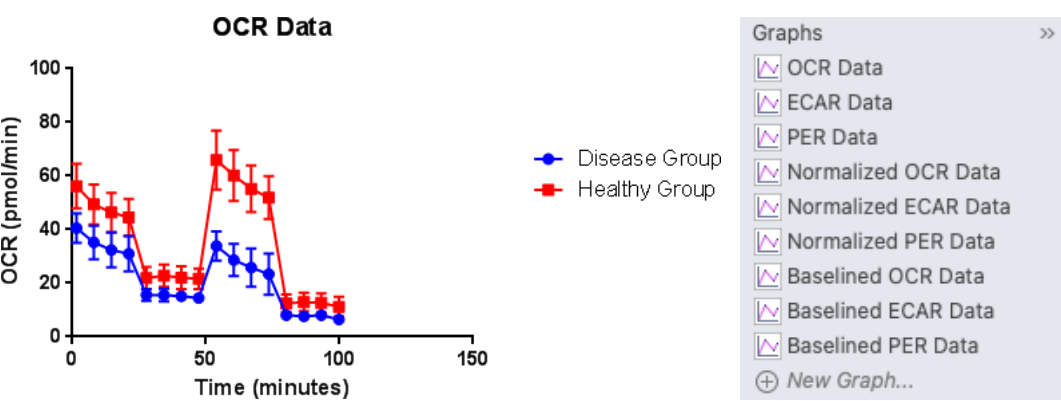


Figure 136. Kinetic graph in Prism export

GraphPad project info

Information about the experiment and assay properties is displayed in Project info 1 (in the Info folder) including the date the assay was performed, the assay result file name, and normalization unit. (See **Figure 137**.) The Notes display on the Project Info view shows the group layout, assay wells in each group, and indicates excluded assay wells using brackets (example: [A2]).

Experiment Date	2/5/2015
Experiment ID	XFp_Seahorse XFp Cell Mito Stress Test_Control vs. Disease
Notebook ID	
Project	
Experimenter	
Protocol	
Background Correction	True
Normalization Scale Factor	5000
Normalization Unit	cells

GROUP LAYOUT

Wells between [ ] are unselected wells and they are not exported in the Data Tables.

Group Names between [ ] are unselected groups and they are not exported in the Data Tables.

Background wells and wells not assigned to a group are not exported in the Data Tables.

Group Name: Disease  
Group Wells: [B1], C1, D1

Group Name: Healthy  
Group Wells: E1, F1, G1

Group Name: Background  
Group Wells: A1, H1

Figure 137. Prism Project info view

## Export to Agilent Seahorse XF Report Generators

XF Report Generators are Microsoft Excel macro files that automatically calculate the parameters of each XF Assay Kit. Result data is presented in a one-page, customizable Summary Report. Wave Pro provides a one-click direct export of result data to the Report Generators. Any modifications in Wave Pro will be incorporated in the exported Report Generator file, such as excluding assay wells from a group and normalized rate data.

If you choose to perform this type of analysis, it is strongly encouraged using **Agilent Seahorse Analytics** over XF Report Generators.

The six XF Report Generators available for direct-export in Wave are:

- XF Real-Time ATP Rate Assay Report Generator
- XF Glycolysis Stress Test Report Generator
- XF Glycolytic Rate Assay Report Generator
- XF Cell Energy Phenotype Report Generator
- XF Cell Mito Stress Test Report Generator
- XF Mito Fuel Flex Test Report Generator



Figure 138. XF Report Generators in the Export menu

### NOTE

**XF Report Generators are validated for compatibility with Microsoft Excel 2010 and later on Windows PCs and Excel for Mac 2011 and 2016.**

**For more information about a specific XF Report Generator, refer to the appropriate Report Generator User Guide on the Agilent website.**

### Export data to an XF Report Generator

- 1 Open assay result file and modify data as necessary.
- 2 Click **Export**.
- 3 Select the XF Report Generator that matches the assay kit you ran.

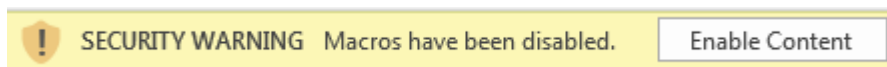
- 4 Enter a file name if desired, select a save location, then click **Save**. The default name of the exported Report Generator file will match the assay result file name.
- 5 Open the **Report Generator** file, and select groups to display. The Select Groups dialogue box is displayed automatically the first time data is exported to the XF Report Generator.
- 6 To save the Summary Report, click **Save**.

NOTE

If an assay result file has data from two different assays on the same cell plate (e.g. XF Real-Time ATP assay data and XF Glycolytic rate assay data), turn OFF groups that do not apply to the Report Generator you are exporting to first. Repeat the same process for the other assay groups and Report Generator.

### Best practices for exporting data to the Agilent Seahorse XF Report Generators

**Enable Macros in the Trust Center** Before using an XF Report Generator, macros must be enabled. Macros are disabled with notification in the Microsoft Excel Trust Center by default. Opening a newly-exported Report Generator will display a security warning to enable the macro. To use the XF Report Generator, click Enable Content. This must be enabled for subsequent XF Report Generator file exports.



To always enable macros and not be prompted to Enable Content each time a Report Generator file is created, enable all macros in the Microsoft Trust Center.

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

**Exporting normalized data to a Report Generator** Normalized rate data in Wave Pro will be exported and used for parameter calculations in the Report Generator. Normalized data is displayed by default after selecting groups to display. To toggle the rate data display between normalized and raw data, use the **Normalize** button on the Summary Printout page. The **Normalize** tab in the Report Generator will display the normalization values, unit, and scale factor as exported from Wave Pro. (See [Figure 139](#) on page 122.) For data integrity purposes,

4 Analyzing Assay Results using Wave Pro  
Export to Agilent Seahorse XF Report Generators

normalization values (displayed on the **Normalize** tab) cannot be edited within the Report Generator. To change normalization values in a Report Generator, you must first edit the normalization values in Wave Pro, then re-export data to the selected Report Generator.

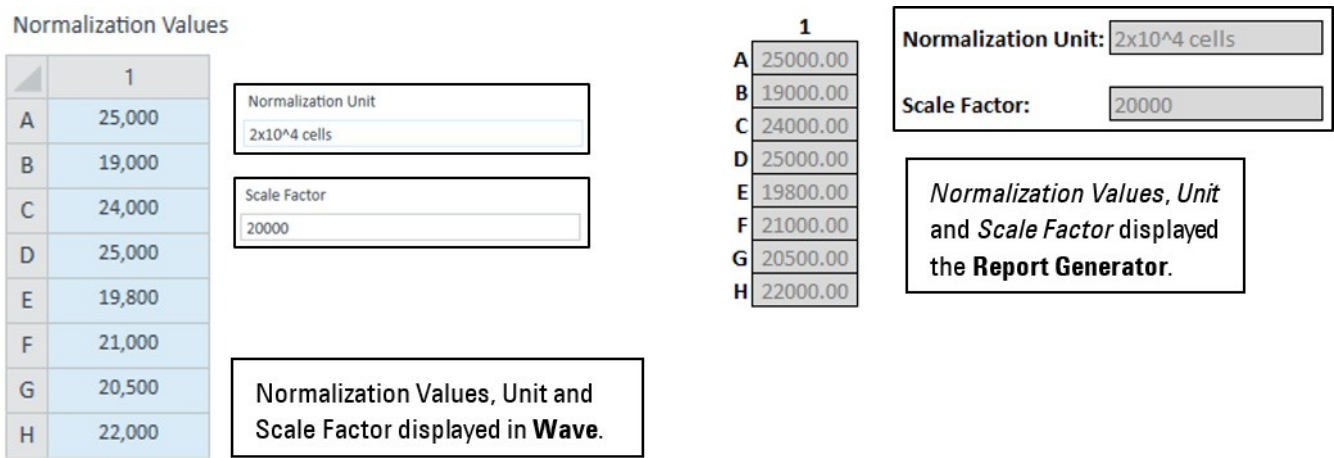


Figure 139. Report Generator normalize tab

The **Normalize** tab in the Report Generators provides a step-by-step explanation on how to edit the normalization values imported to the Report Generator. (See [Figure 140](#).)

**Note:** Normalization values in the Report Generator are automatically imported and applied from the Wave Desktop Normalization Plate Map. To modify these normalization values:

1. Open the Assay Result file in Wave Desktop.
2. Edit values in the Normalization Plate Map in Wave Desktop.
3. Export result data to the desired Report Generator.

Figure 140. Instructions to edit the normalization values

**Excluded assay wells in Report Generator** Data from assay wells (or entire groups) that are turned off in Wave Pro will not be exported / included in parameter calculations in the Report Generators. The Measures Sheet displays the group names, Plate Map layout, and any assay wells that have been excluded in the group calculations in the Report Generator. (See [Figure 141](#).)

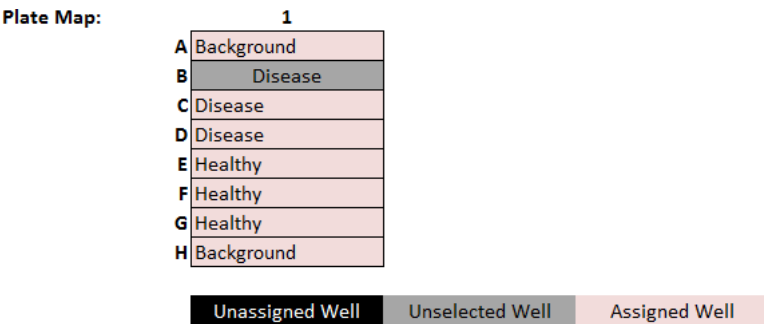


Figure 141. Measures sheet

## Wave Pro Summary View

The Summary view is always displayed as the first-listed analysis view in the Views section on the ribbon (next to Add View). Every assay result file contains the Summary view, this view cannot be deleted. The purpose of the Summary view is to record important notes or information about the assay result file. You may also append graphs, charts, Plate Maps from analysis views in the file, or add details of the Instrument Protocol or Groups/Conditions used in the assay. The Summary is the only location in Wave Pro that enables export to Microsoft Word, Adobe PDF, HTML, or Rich Text Format (.rtf). (See [Figure 142](#).)

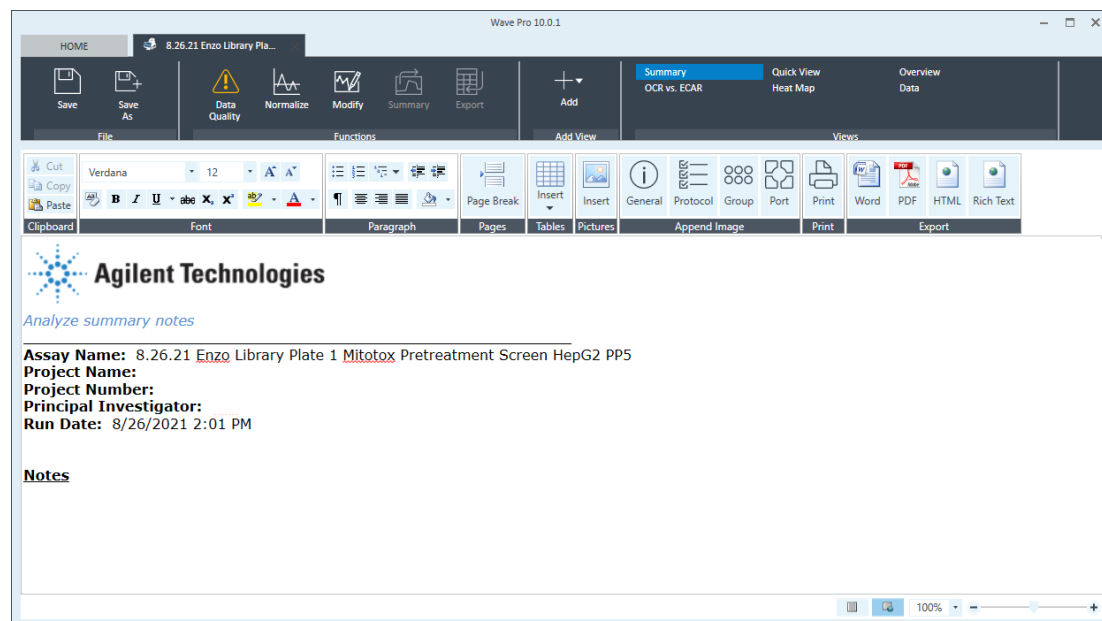


Figure 142. Summary view

## Customize the summary

Use the ribbon toolbar to add, edit, and format the content added to the Summary, such as:

- Cut, copy, and paste to the clipboard.
- Change the font type, size, text, highlight color, and style.
- Subscript or superscript a character or characters.
- Add bullet points or numbers.
- Insert a table, picture or page break.

### Append to summary

Append content from any analysis view directly to the Summary in three ways:

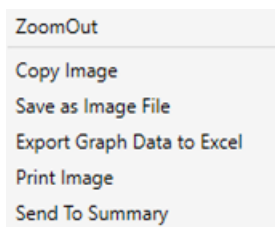
- Append to Summary: Click Summary (in the Functions ribbon) on an open analysis view to send all data from the current analysis view to the Summary page.



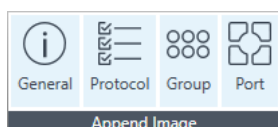
## 4 Analyzing Assay Results using Wave Pro

### Customize the summary

- Right-click > Send to Summary: Right-click a kinetic graph, scatter plot, or Plate Map and click Append to Summary from the drop-down list. To view appended content, click the Summary view.



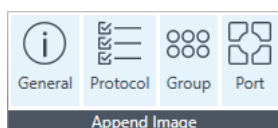
- Append Image (Summary view)
- General Information: The General Information button appends an image to the Summary view containing information about the assay, including project and plate information.



### Assay Summary

Project Information	Plate Information	Notes
Project Name <input type="text"/>	Well Volume (μl) <input type="text" value="2.28"/>	<div></div>
Principal Investigator <input type="text"/>	Plated By <input type="text"/>	
Project Number <input type="text"/>	Plated On Select Date <input type="text" value="15"/>	
Show Advanced Options <input type="checkbox"/>		

- Protocol Summary: The Protocol Summary appends the Instrument Protocol (Mix, Wait, and Measure steps) as well as the time and number of cycles for each measurement to the Summary view.



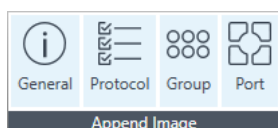
Summary				
Protocol				
Total Time: 01:24:00				
Group				
Port A				
Port B				
Port C				
Port D				
Initialization	Baseline	Oligomycin	FCCP	Rotenone + Antimycin A
Calibrate	Mix: 00:03:00	Inject Port: A	Inject Port: B	Inject Port: C
Equilibrate: 00:12:00	Wait: 00:00:00	Mix: 00:03:00	Mix: 00:03:00	Mix: 00:03:00
	Measure: 00:03:00	Wait: 00:00:00	Wait: 00:00:00	Wait: 00:00:00
	Mix: 00:03:00	Measure: 00:03:00	Measure: 00:03:00	Measure: 00:03:00
	Wait: 00:00:00	Mix: 00:03:00	Mix: 00:03:00	Mix: 00:03:00
	Measure: 00:03:00	Wait: 00:00:00	Wait: 00:00:00	Wait: 00:00:00
	Mix: 00:03:00	Measure: 00:03:00	Measure: 00:03:00	Measure: 00:03:00
	Wait: 00:00:00	Mix: 00:03:00	Mix: 00:03:00	Mix: 00:03:00
	Measure: 00:03:00	Wait: 00:00:00	Wait: 00:00:00	Wait: 00:00:00
		Measure: 00:03:00	Measure: 00:03:00	Measure: 00:03:00



## 4 Analyzing Assay Results using Wave Pro

### Print and Export summary

- **Group Summary:** The Group Summary button appends an image of the Plate Map and a table of the Group Definitions to the Summary view. The Plate Map is shown below.

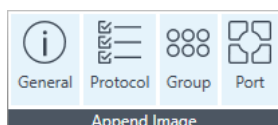


### GROUP Summary

Plate Map

B	2	2	8	8	8	4	4	4		6	8
2	2	2	8	8	8	4	4	4			
2	2	2	8	8	8	4	4	4		6	6
2	2	2	8	8	8	4	4	4			
1	1	1	7	7	7	3	3	3	5	5	5
1	1	1	7		7	3	3	3	5	5	5
1	1	1	7	7	7	3	3	3	5	5	5
B	1		7	7	7	3	3	3	5	5	B

- **Port Summary:** The Port Summary button appends an image to the Summary view of the contents injected from each compound port in the assay.



### Port A Summary

Plate Map

B	1	1	2	2	2	2	2	1	1	B
1	1	1	2	2	2	2	2	1	1	1
1	1	1	2	2	2	2	2	1	1	1
1	1	1	2	2	2	2	2	1	1	1
1	1	1	1	1	1	2	2	1	1	1
1	1	1	1		1	2	2	1	1	1
1	1	1	1	1	1	2	2	1	1	1
B	1		1	1	1	2	2	1	1	B

Port A



Volume: 25 µl

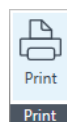
8 Background Well

- 1 Oligomycin  
Concentration: 1 µM  
Solvent: 100 % assay media
- 2 Glucose  
Concentration: 0  
Solvent: 0 %

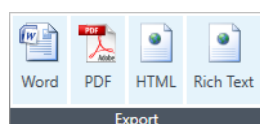
Friendly reminder: Make sure you fill the ports of your Background Wells.

## Print and Export summary

To print the Summary view, click the **Print** button on the **Summary** toolbar, select the printer to use, and click **Print**.



Export the contents of the Summary view to a Microsoft Word Document, PDF file, HTML file, or Rich Text Format file. Click the appropriate icon from the Export section of the Summary toolbar, browse to the desired file location to save and click Save.



## Data View

The Data view provides access to all data associated with an assay result file.

Data is organized into 7 tabs:

- **Group Data:** OCR, ECAR, and PER data and error values organized by group, and ordered by measurement.
- **Rate:** OCR, ECAR, and PER individual assay well data. Error values are not displayed.
- **Level Data:** Individual assay well data displayed as Level data - O2 level (in mmHg) and pH raw values.
- **Raw:** Raw data values obtained during the assay including LED values, temperature at each measurement, and internal LED reference values.
- **Calibration:** Calibration results for each assay well are displayed in a table format.
- **Calibration View:** Calibration results for each assay well are displayed in a Plate Map format.
- **Event Log:** Analyzer serial number, consumable lot numbers used during assay, a command log for processes performed during assay, and the licenses status of the result file: XF Discovery, Assay Kit info, and validation status..

Export the data in a selected tab to Microsoft Excel using the right-click **Export** function. The Calibration, Raw, and Event Log tabs are used primarily for internal purposes, including QC, validation processes, and Agilent Cell Analysis Technical Support.

To display the Data view, click **Add View** on the top-level ribbon and select **Data** from the drop-down menu. The **Group Data** tab is displayed by default when opening the Data view. (See [Figure 143](#).)

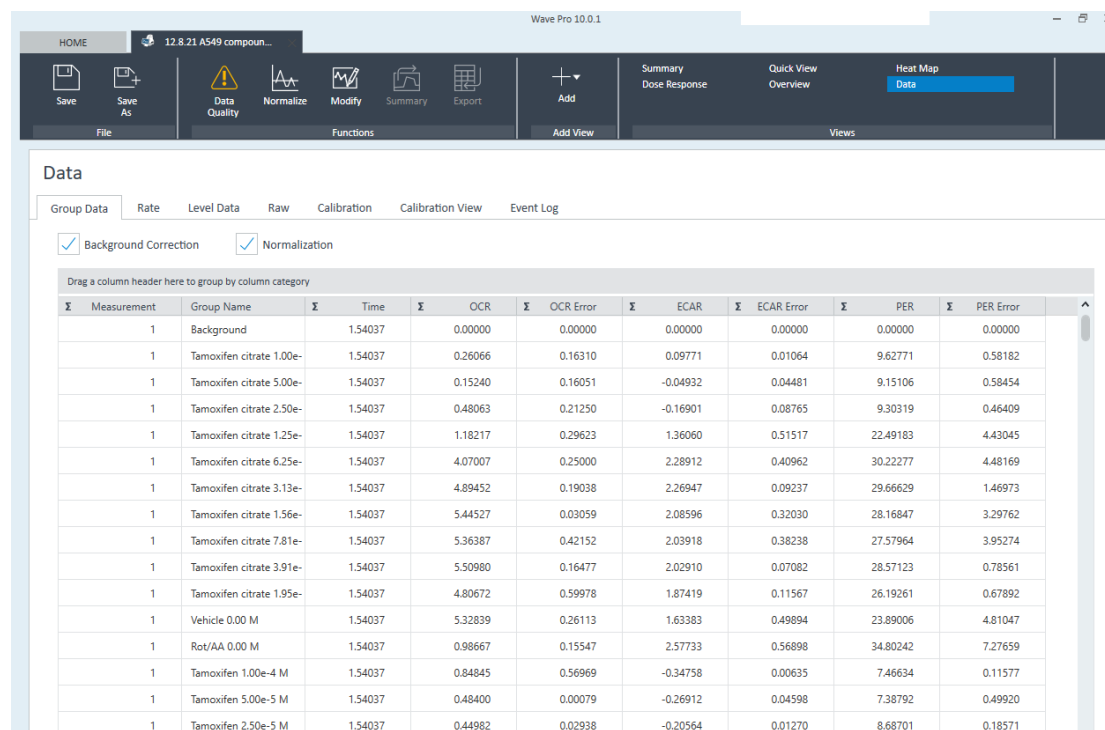


Figure 143. Data view

## Column sorting

To sort the data by a specific column, click on the column header: Measurement, Group Name, Time, Rate or error for OCR, ECAR, or PER. Wave Pro will automatically sort the data in ascending or descending order in the selected column.

Data

Group Data

Rate

Level Data

Raw

Calibration

Calibration View

E

Background Correction

☒

Normalization

☒

Drag a column header here to group by column category

<div>Σ</div>	Measurement	Group Name	<div>Σ</div>	Time	<div>Σ</div>	OCR
	1	Background		1.36576		0.00000
	1	Glyco-MCF10A Par		1.36576		10.65762
	1	Glyco-MCF10A Par no e		1.36576		9.31720
	1	Glyco-MCF10A E545		1.36576		7.02602
	1	Glyco-MCF10A H1047		1.36576		7.31512
	1	Mito-MCF10A Par		1.36576		10.20519
	1	Mito-MCF10A Par no eg		1.36576		8.70299
	1	Mito-MCF10A E545		1.36576		8.00559
	1	Mito-MCF10A H1047		1.36576		7.45769

## Display values for: Average, Count, Maximum, Minimum, or Sum

To display the Average, Count, Maximum, Minimum, or Sum, or any combination of these values, click the **Sigma** sign to the right of the arrow, and check the applicable boxes. In the example shown below, all values are checked for OCR data - Wave Pro will display the average, count, maximum value, minimum value, and sum for the OCR data.

<div>Σ</div>	Time	<div>Σ</div>
		<div><div><div><input type="checkbox"/></div>Average</div><div><input type="checkbox"/></div>Count</div> <div><input type="checkbox"/></div> Maximum

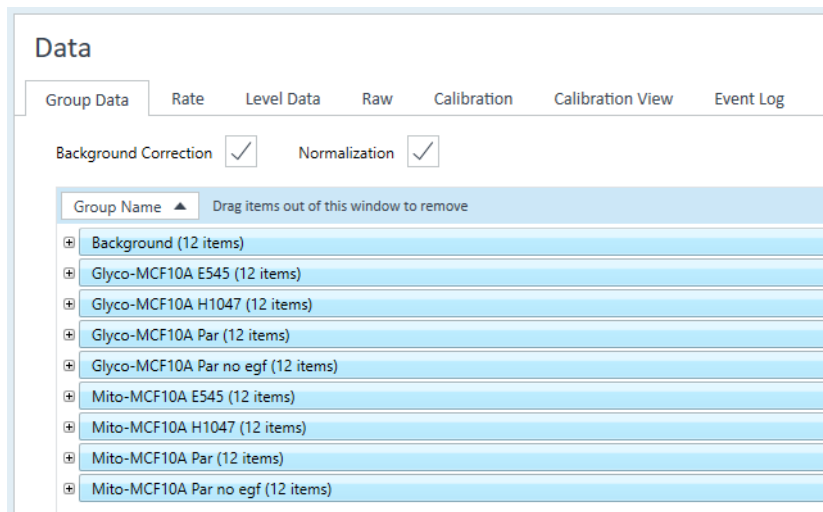
☐☐

☒

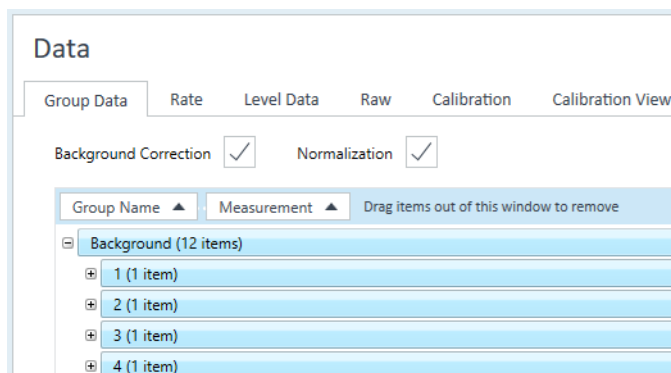
☒

## Group Data by Field

To group the data by field, drag and drop a column header to the gray bar above the table. In the example shown below, the Group Name heading was dragged to the gray bar. After grouping the data, expand or collapse the group by clicking on the plus/minus sign to the left of the group.



After expanding a group, select another column heading to define the groupings further. In the example below, the heading OCR was dragged to the grey bar after Group Name.



## Managing Agilent Seahorse Files

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## Wave Pro & XF Pro Controller: Templates view

Manage the list of available templates for your XF Pro and/or XF HS Mini Analyzer from the Templates view, **Figure 144**.

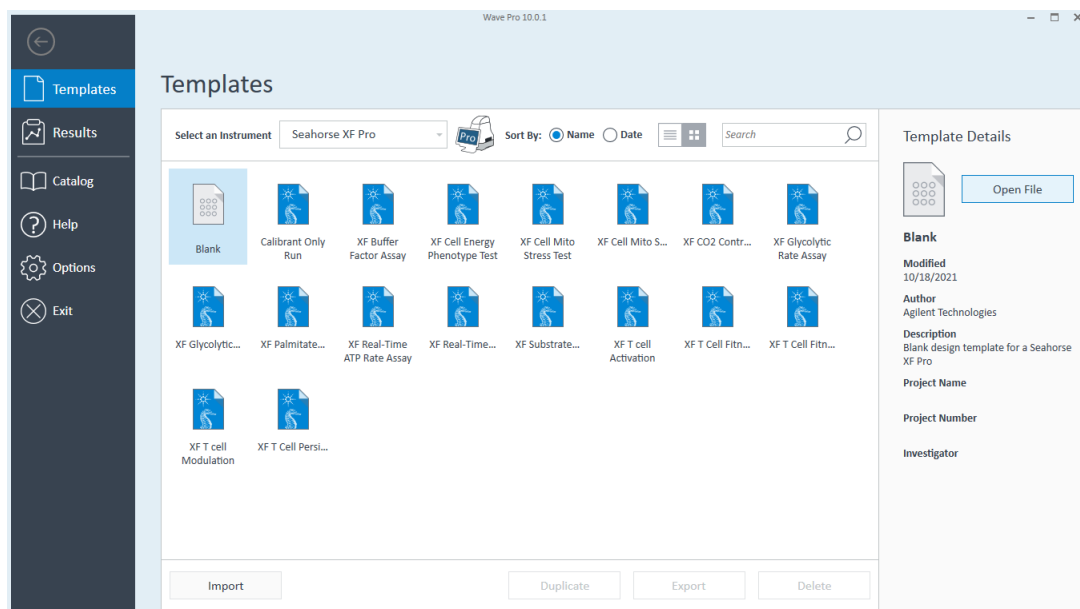


Figure 144. Wave Home > Templates view

### Import assay templates

Import assay template files to Wave Pro and XF Pro Controller software:

- Double-click the assay template file, and the template will automatically import to the Templates view in Wave Pro / XF Pro Controller software.
- Click Import on the Templates view, locate the assay template file, then click **Open**.

### Export, duplicate, and remove assay templates

Select (single-click) an assay template on the Templates view to:

- **Duplicate:** Create an identical copy of a template.
- **Delete:** Permanently delete a template.
- **Export:** Export a template to:
  - A USB flash drive
  - A shared network directory for multiple user access

## Template Details

To display details about an assay template without opening the file, single-click the template to display Template Details. (See [Figure 145](#).) The Modified date reflects the most recent date the template was modified and changes were saved. Description, Project Name, Project Number, and Investigator are optional details and not required to save a template.

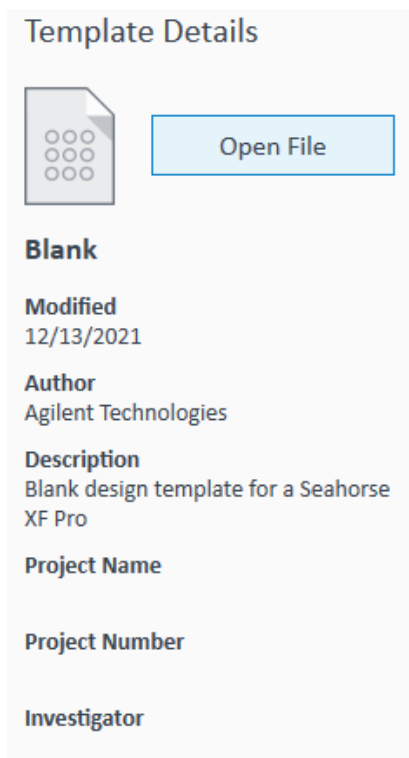


Figure 145. Template Details

# Wave Pro (only): Results view

The Results view, **Figure 146**, displays recently opened assay result files, the corresponding file directory where each assay result file is located, and Favorite Places. The Results view is a feature of Wave Pro software only.

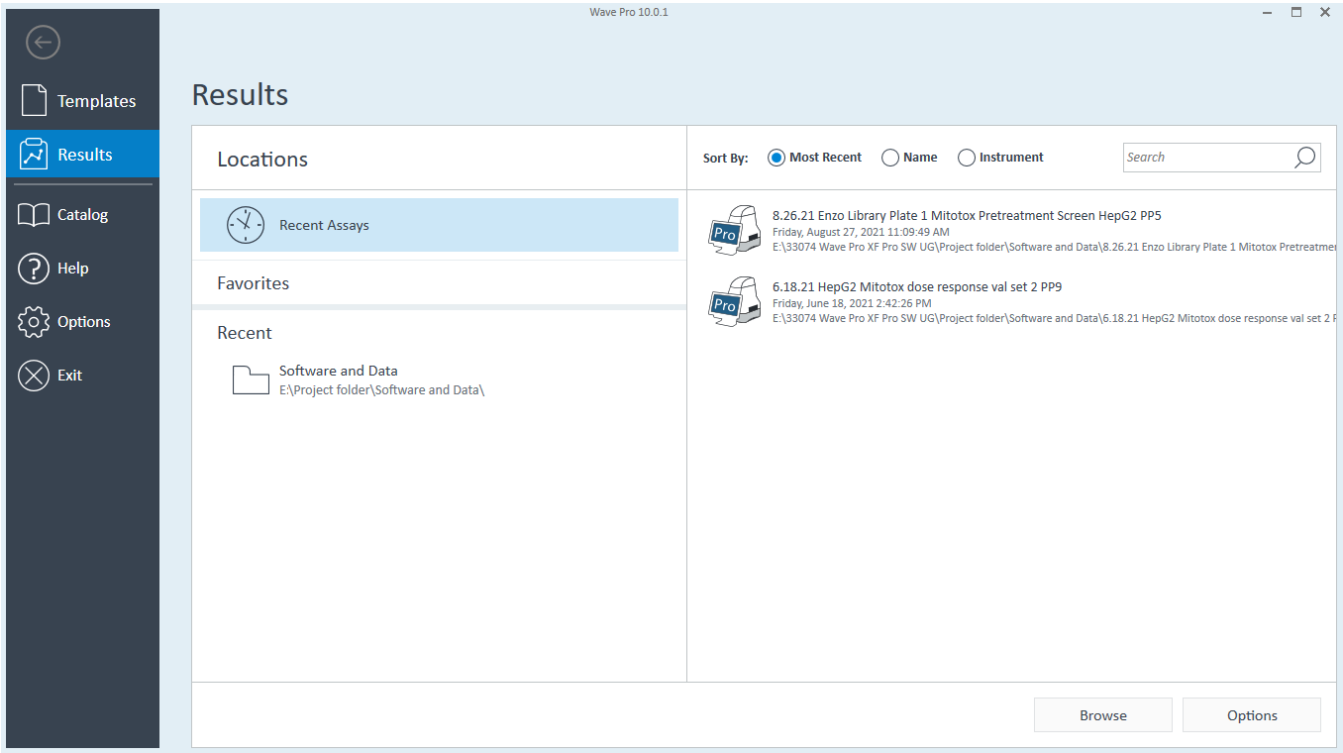


Figure 146. Wave Home > Results view



## Recent assays, recent places, and favorite places

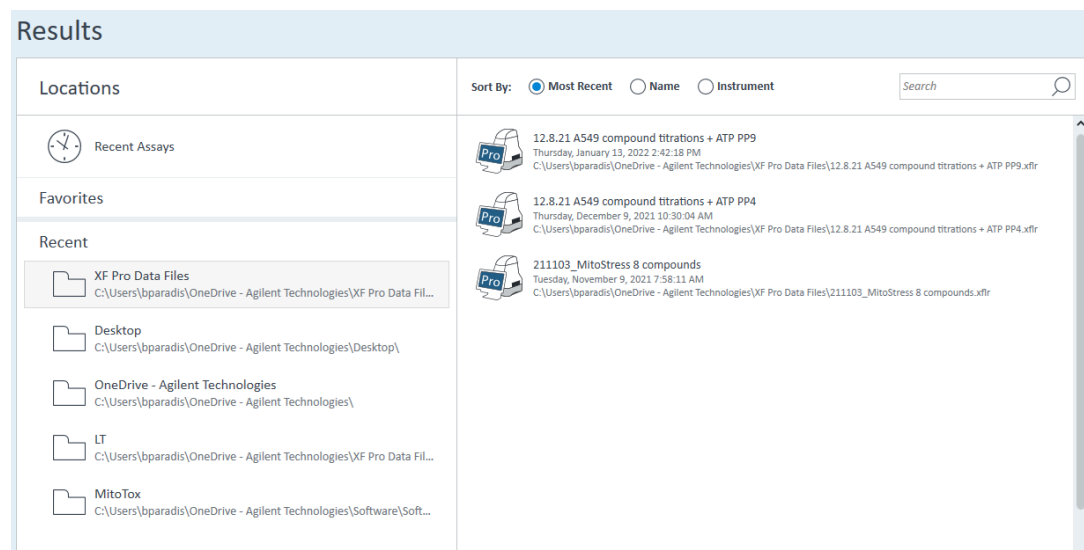


Figure 147. Recent Assays, recent places, and favorite places

- Recent Assays displays a sortable and searchable list of recently viewed and analyzed assay result files. (See [Figure 146](#).)
- Favorites displays user-defined locations (local or network directories) where assay result files are frequently added to, or accessed from. Once configured, each local directory added as a Favorite Place will appear below Recent Assays as a folder icon. (See [Figure 147](#).)
- Recent displays the file location (for both local and network directories) of recently opened assay result files in the order of when each result file was opened. (See [Figure 147](#).)

## Options

Click Options, next to Browse in [Figure 146](#), to add Favorite Places (that is, a shared network directory) or configure the number of Recent Folders and Recent Assay Files to display on the Results view. (See [Figure 148](#).)

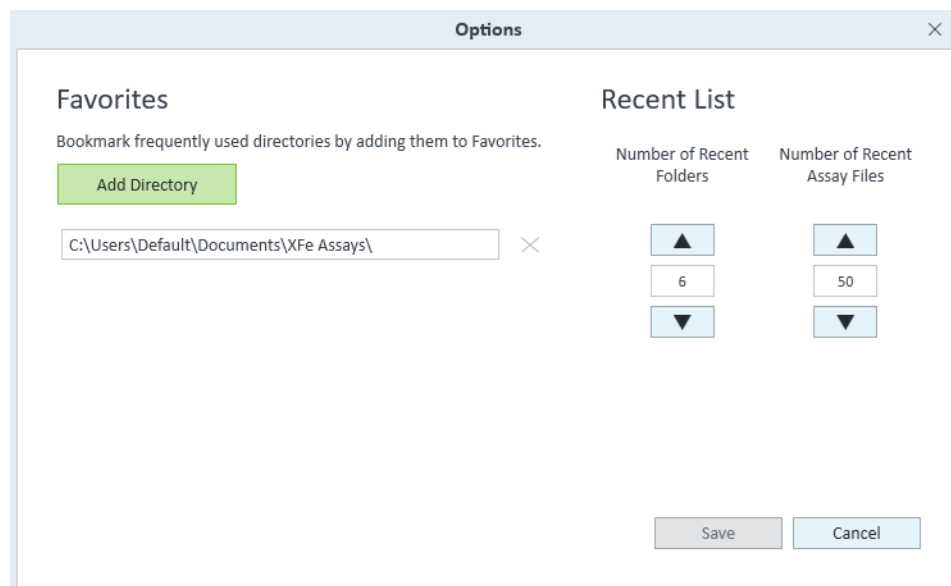


Figure 148. Edit result file display options

## Browse

To manually open an assay template or assay result file, click Browse. (See [Figure 146](#) on page 132.) The file types compatible with Wave Pro are:

- Assay result files (\*.xflr)
- Assay templates (\*.asyt)

File types compatible with XF Pro Controller software are assay templates (\*.asyt only). Assay result files (\*.xflr) cannot be opened / viewed using XF Pro Controller software.

There are two additional Agilent Seahorse assay result file types:

- Agilent Seahorse XFe & XFp assay result files (\*.asyr)
- Agilent Seahorse XF24 and XF96 assay result files (\*.xfd)

These result file types are not compatible with Wave Pro & XF Pro Controller software and must be opened using Agilent Seahorse Analytics or Agilent Seahorse Wave Desktop software

## Sort by

Assay result files are default-sorted by Most Recent, which displays a list of the most-recently opened or modified assay result file first. Name sorts assay result files alphabetically based on the file name and Instrument sorts by the type of XF Analyzer used to generate the assay result file.

Sort By: ☒ Most Recent ☐ Name ☐ Instrument

Figure 149. Results file sorting options

## Search

Type specific keywords in the Search bar to filter and display assay result files whose file name contains matching keywords. Searching using the file directory or date is not supported.

A rectangular search bar with a light gray border. Inside the bar, the word "Search" is written in a blue, sans-serif font. To the right of the text, there is a magnifying glass icon, also in blue.

Figure 150. Search from the results view

## Wave Pro & XF Pro Controller: Catalog view

The Catalog provides a location to save frequently used Compounds, Pretreatments, Media, and Cells. (See [Figure 151](#).) Save time when creating a new assay template or customizing a template file by inserting Catalog entries. The Catalog is prepopulated with reagents and compounds that are used in commercially available Agilent Seahorse Assay Kits.

Catalog						
	Name	Reagent Name	Port Concentration	Port Concentration Unit	Solvent	Solvent Percentage(%)
Compounds	Seahorse XF Glycolysis Stress Test-1	Glucose	0.00	▼		0.00
	Seahorse XF Glycolysis Stress Test-2	oligomycin	0.00	▼		0.00
	Seahorse XF Glycolysis Stress Test-3	2-DG (deoxy-D-glucose)	0.00	▼		0.00
Pretreatments	Seahorse XF Mito Fuel Flex Test - Fatty Acid Inhibitor	etomoxir	0.00	▼		0.00
	Seahorse XF Mito Fuel Flex Test - Glucose Inhibitor	UK5099	0.00	▼		0.00
	Seahorse XF Mito Fuel Flex Test - Glutamine Inhibitor	BPTES	0.00	▼		0.00
Media	Seahorse XF Mito Stress Test-1	oligomycin	0.00	▼		0.00
	Seahorse XF Mito Stress Test-2	FCCP	0.00	▼		0.00
	Seahorse XF Mito Stress Test-3	Rotenone-Antimycin A	0.00	▼		0.00
	Seahorse XF Phenotype Test-1	oligomycin	0.00	▼		0.00
	Seahorse XF Phenotype Test-2	FCCP	0.00	▼		0.00

Figure 151. Wave Home > Catalog view

### Add a catalog entry

- 1 Open the Catalog view.
- 2 Select a condition (Compounds, Pretreatments, Media, and Cells). This example used Pretreatments.
- 3 At the bottom of the Catalog > Pretreatments view, type in a Name (required), and any additional fields to describe the Pretreatment entry (optional).
- 4 After adding details, click Add Pretreatment to save the entry.
- 5 When finished, click Save in the lower-right corner of the Catalog view.

### Delete a catalog entry

- 1 Open the Catalog view.
- 2 Click the red X at the end of the row to delete.

## Wave Pro & XF Pro Controller: Options view

The three types of modifiable settings in the Wave Pro Options view are: General, Instrument, and Advanced. (See [Figure 152](#).) There are two additional options available in XF Pro Controller software, they are **Automation** and **Licenses** (not pictured in [Figure 152](#)).

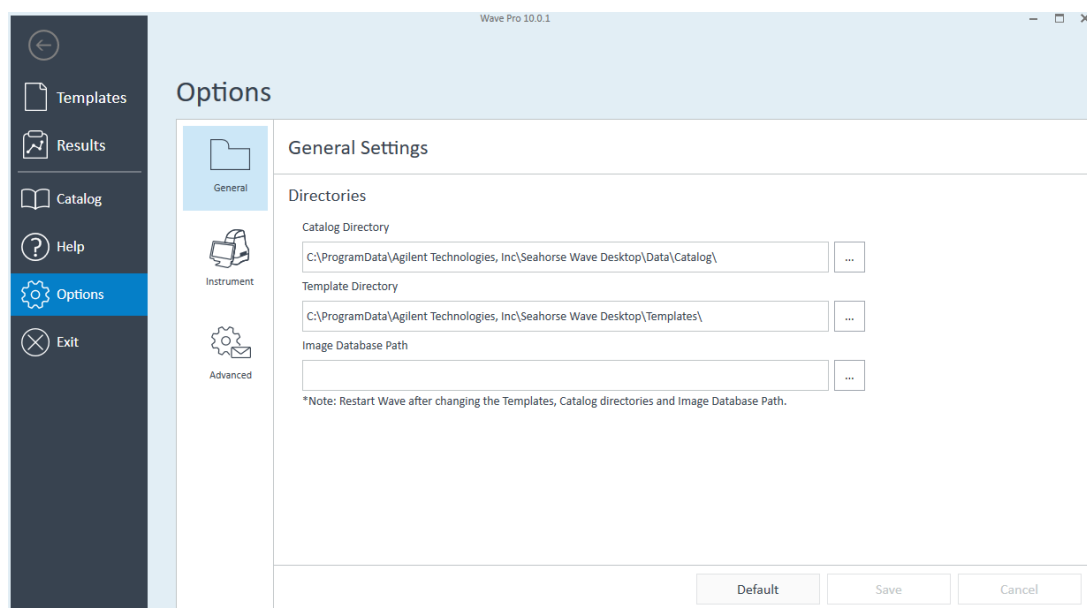


Figure 152. Wave Pro Home > Options view

### General settings

General settings provides options to:

- View or edit the catalog and template directories (default save locations), view or edit the Image Database Path.
- Change default instrument settings (e.g., default Measure time)
- Enable/disable email notifications
- Claim Seahorse software licenses (XF Pro Controller only)

After making changes to General options, click Save (lower-right corner) to save changes. To restore changes to the factory default setting, click the Default button.

#### Directories

Directories displays the default save location for Catalog entries and Template files. (See [Figure 104](#).) The prepopulated Catalog conditions and assay template files installed with Wave Pro and XF Pro Controller software are stored in these directory locations, as well as any new Catalog entries and assay template files. To change the locations, click the [...] button to browse to the preferred location. Restart Wave Pro / XF Pro Controller for changes to take effect.

## Instrument options

Instrument options displays default Instrument Protocol command values (by analyzer type). (See [Figure 153](#).)

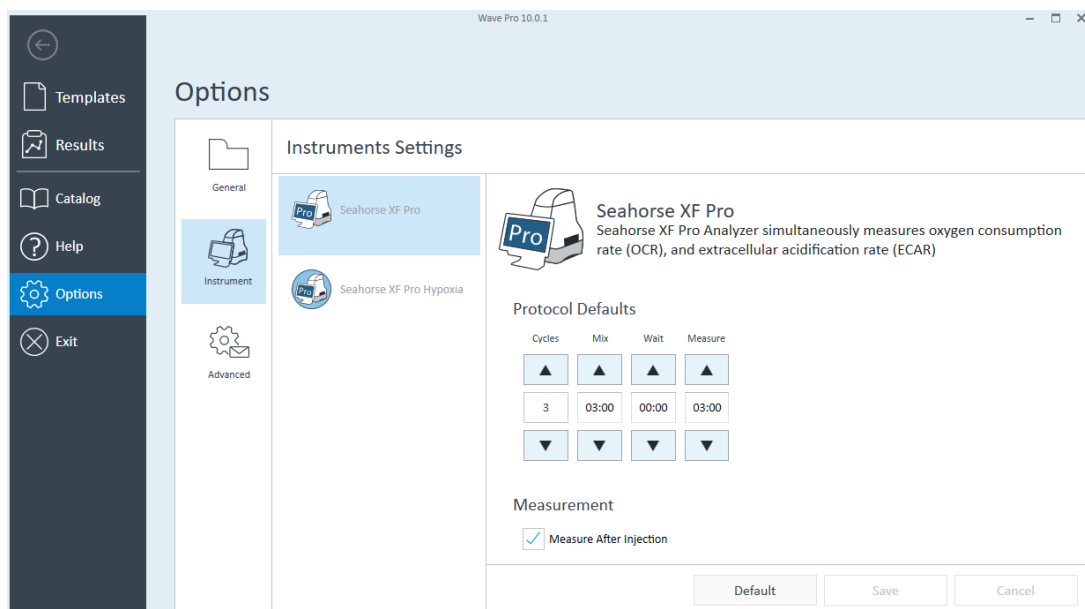


Figure 153. Instrument options

Other settings that can be modified include:

- **Cycles:** Number of repeat **Mix**, **Wait**, and **Measure** cycles in the default protocol
- **Mix**, **Wait**, and **Measure** times: Time to complete each command in the Instrument Protocol
- Change atmospheric pressure value (for calculation purposes; setting accessible in XF Pro Controller software only).

### Protocol defaults

Leave the default **Protocol Defaults** untouched, and modify the Instrument Protocol in the individual assay template file. To change the **Protocol Defaults**:

- 1 Use the up/down arrows or type in the new values for **Mix**, **Wait**, **Measure**, and **Cycles**.
- 2 Click **Save** at the bottom of the screen to save changes.

## Advanced options (XF Pro Controller software only)

XF Pro Controller software (for XF Pro Analyzers only) can be configured to automatically notify users when certain functions are complete or require attention, such as:

- When calibration has finished and it is time to replace the Calibration Plate with the Cell Plate and start the assay.
- When your assay completes, XF Pro Controller software will automatically email the assay result file to the specified email addresses.

## 5 Managing Agilent Seahorse Files

### Advanced options (XF Pro Controller software only)

An active internet connection on the XF Pro Analyzer is required for use of automatic email notification features. (See [Figure 154](#).)

Wave Pro 10.0.1

Options

Automated Email

General

Instrument

Advanced

Sender

Mail From

test@mydomain.com

Password

SMTP Address

smtp.server.com

Port

0

Enable SSL

Test Settings ...

Recipients

Recipient Email Address

test@theirdomain.com

Add

Email Recipients

Default

Save

Cancel

Figure 154. Automated Email features

#### Add email recipients

- 1 Type an email address in the **Mail From** field, and enter a password for this address in the **Password** field. This email address will be used to send all notifications and will be displayed in the **Mail From** field after receiving a notification from the XF Pro Analyzer.
- 2 Specify the **SMTP Address** and the access **Port** field.
- 3 Check **Enable SSL** if required by the local IT group.
- 4 Type the email address for a single recipient in the **Recipient Email Address** field.
- 5 Click **Add**.
- 6 Repeat Steps 4 and 5 for each recipient.
- 7 When finished adding recipients, click **Save**.
- 8 To send a test notification email to all email recipients, click **Test Settings**. If the test email is not received, verify the correct network settings have been configured in XF Pro Controller software.

#### Remove email addresses

Select the email address under **Email Recipients**, and click **Delete**.

## Automation (XF Pro Controller software only)

The **Options > Automation** view is where to activate the automation service for your XF Pro Controller for purposes of automating XF assays & data acquisition.

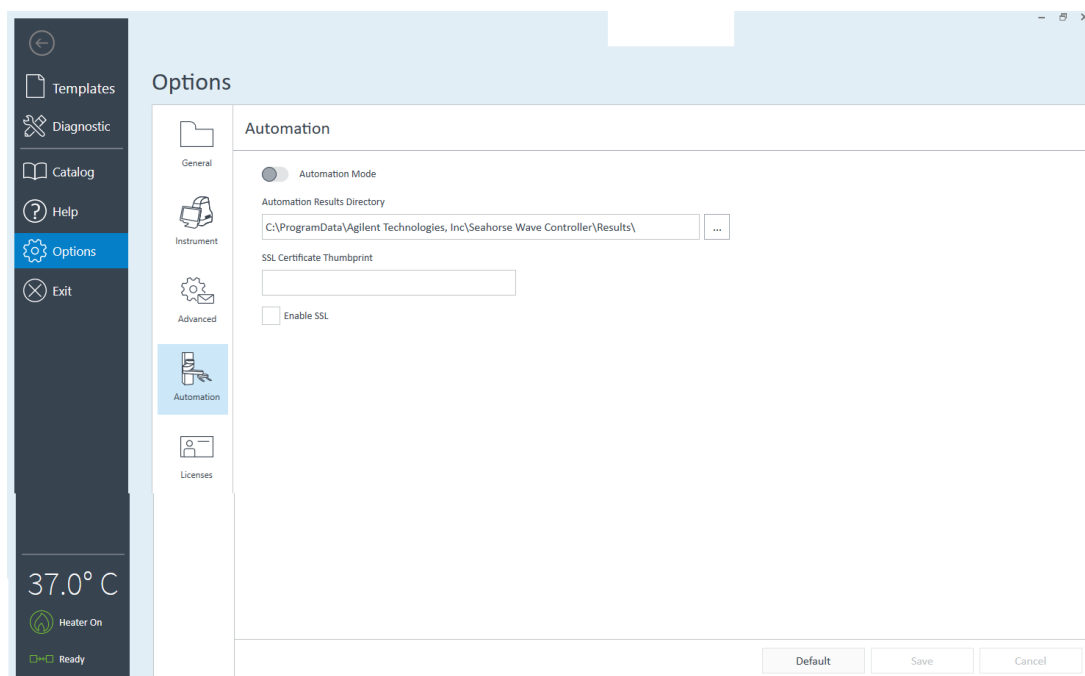


Figure 155. Options > Automation view

This feature can only be activated in XF Pro Controller software with a valid XF Discovery license. To enable the automation service, toggle **Automation Mode** ON (**Figure 155**). Once Automation Mode is enabled, XF Pro Controller software is ready to begin receiving method calls from a client application interface. To learn more about this capability, please contact your local Agilent Seahorse Product Specialist, or the Agilent Cell Analysis Support team at: [cellanalysis.support@agilent.com](mailto:cellanalysis.support@agilent.com)



## Licenses (XF Pro Controller software only)

The **Options > Licenses** view is where to manage your software licenses for your XF Pro Controller. For more information on the XF Discovery license, including steps to claiming and activating the license, please see **“Claiming your Agilent Seahorse XF Discovery License using XF Pro Controller software”** on page 60.

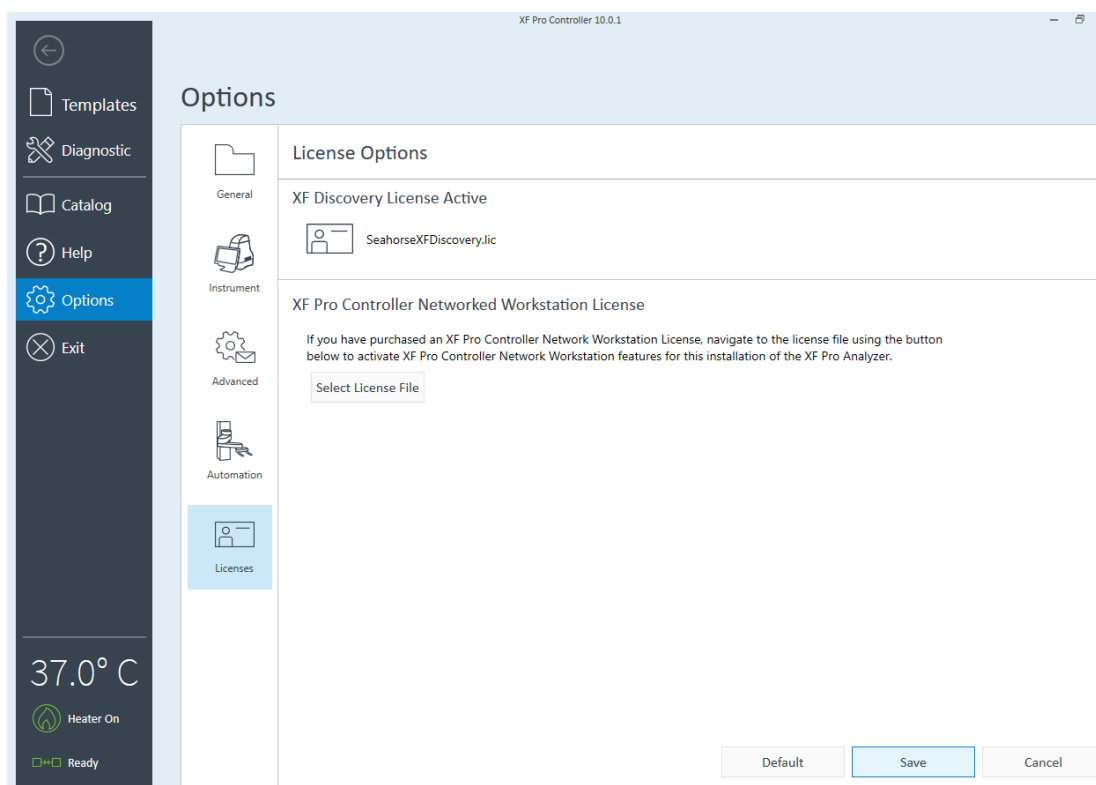


Figure 156. Options > Licenses view

## XF Pro Controller (only): Diagnostics

The XF Pro Controller **Diagnostic** view (**Figure 157**) contains various functions that assist Agilent Field Service Engineers and Cell Analysis Technical Support in debugging any issues or errors encountered during normal operation. Perform the **System Check** diagnostic function at initial installation of the XF Pro Analyzer and when Cell Analysis Technical Support request to do so. The Diagnostic view provides the following sections:

- Instrument QC
- QC Results
- System Check
- Consumable Check
- Maintenance

### Instrument QC

The XF Pro QC Kit diagnostic test is for trained Agilent Field Service Engineers only.

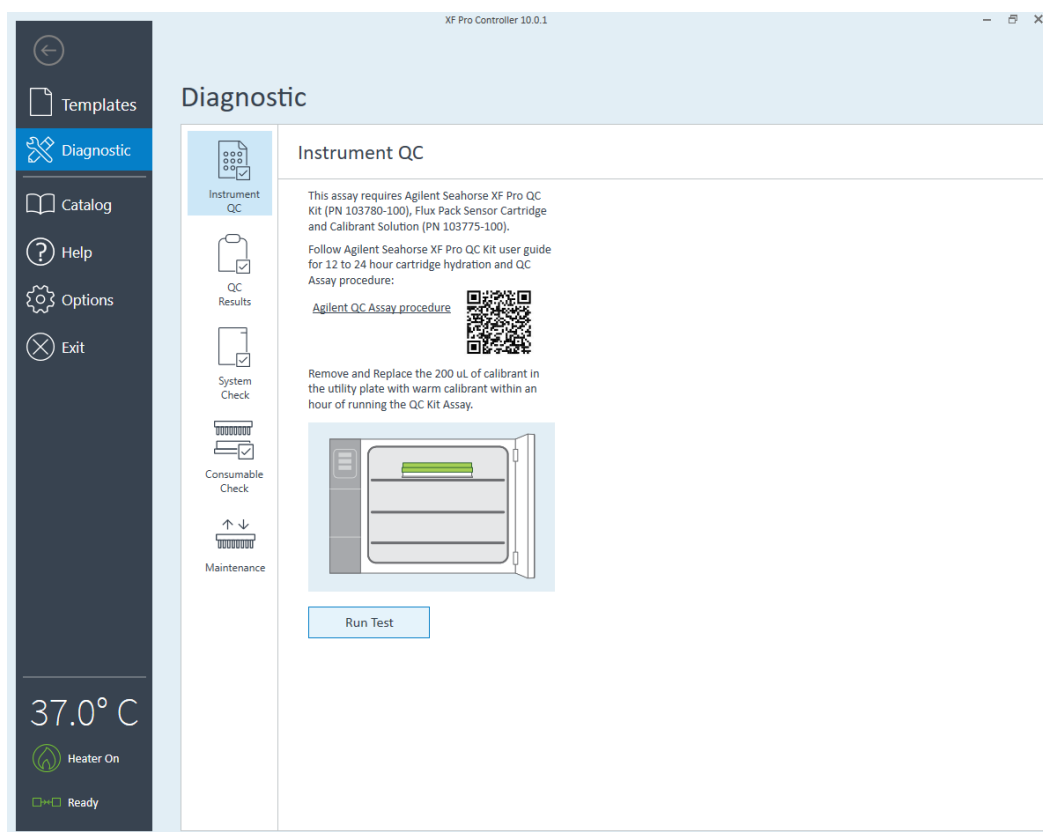


Figure 157. Diagnostic > Instrument QC view

## QC Results

This diagnostic function shows all available XF Pro QC Kit result file test history, information about each result file, export options, and more.

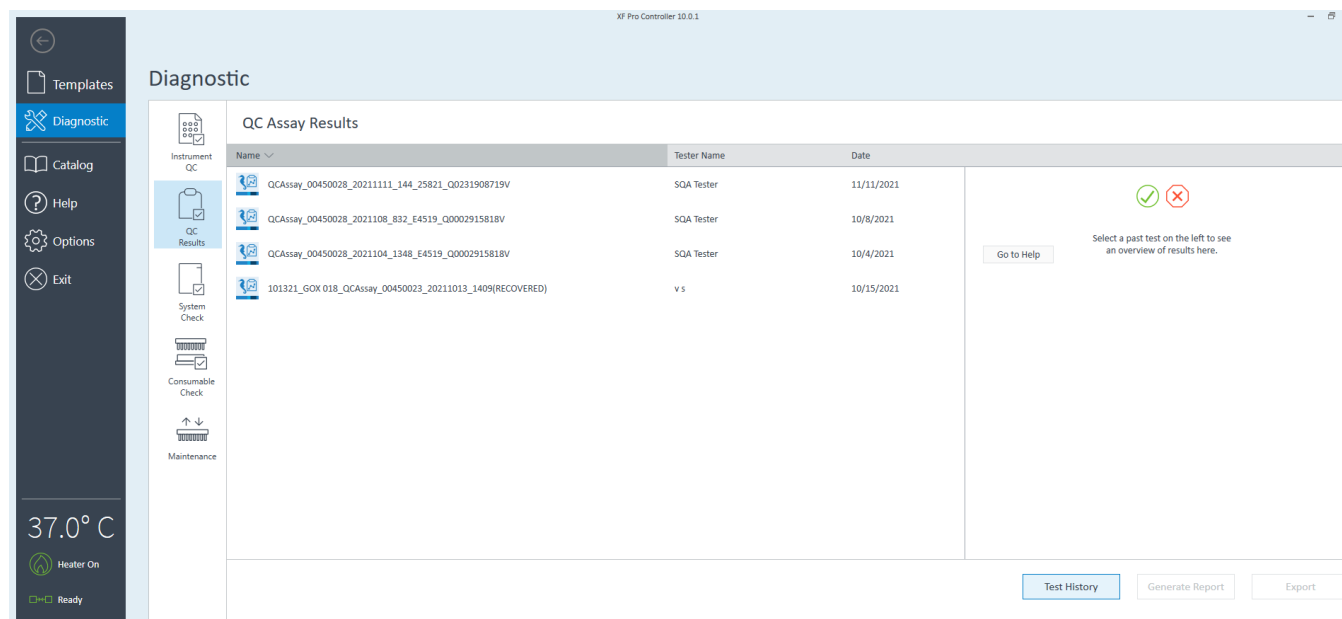


Figure 158. Diagnostic > QC Results view

## System Check

### Overall System:

- **Communication test** - Verifies all system modules are communicating appropriately.
- **Voltage test** - Ensures voltage levels are within specified ranges for operation.
- **Motor test** - Verifies the movements and positions of the tray and probes.
- **Holding pressure test** - Ensures the injection system does not have any leaks.
- **Injection solenoid test** - Verifies proper open/close operation of internal valves.

### Measurement System:

- **LED reference test** - Verifies that the system records LED intensity signal when ON.
- **Autozero test** - Analyze and record any offset values found within the channels on each board.
- **Noise test** - Analyze and record the amplitude of the noise level on each channel.
- **High-Gain rise time test** - Conduct an amplifier rise time test.

### Thermal Performance:

- **Thermal test** - Verifies that the temperature remains within tolerance after a "Wait" period.

## 5 Managing Agilent Seahorse Files

### Consumable Check

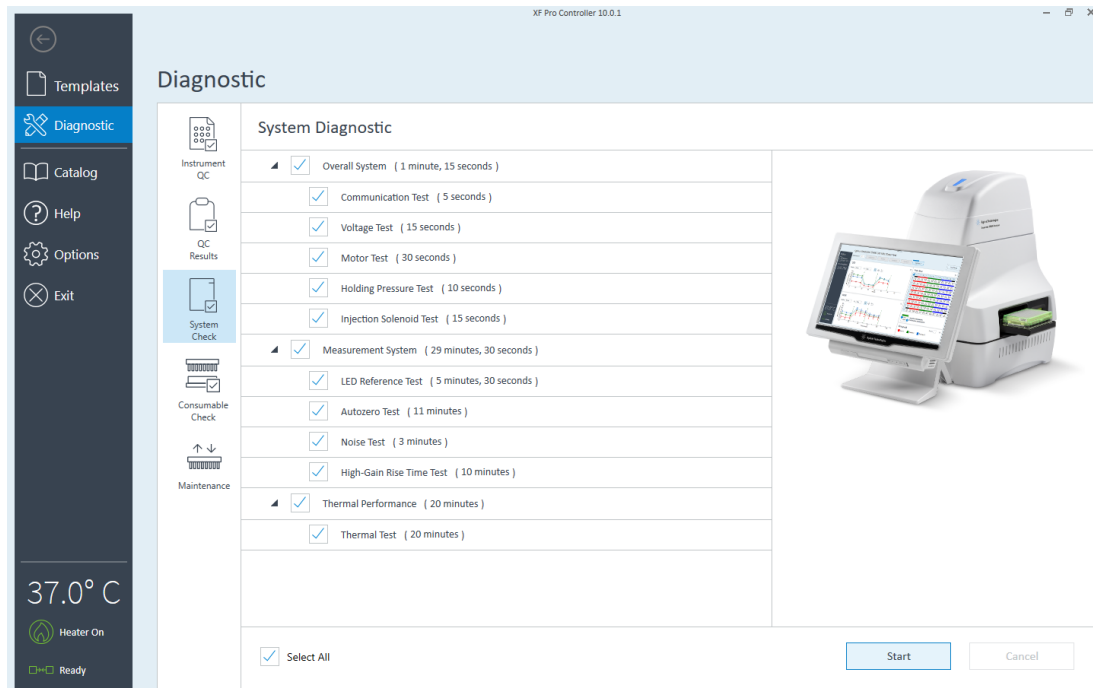


Figure 159. Diagnostic > System Check view

## Consumable Check

- **Barcode test** - Verifies XF Pro Analyzer can read barcodes (requires user interaction to complete).
- **Calibration test** - Performs calibration procedure and will verify that all wells are calibrated correctly.
- **Injection test** - Verifies proper performance of injection mechanism.

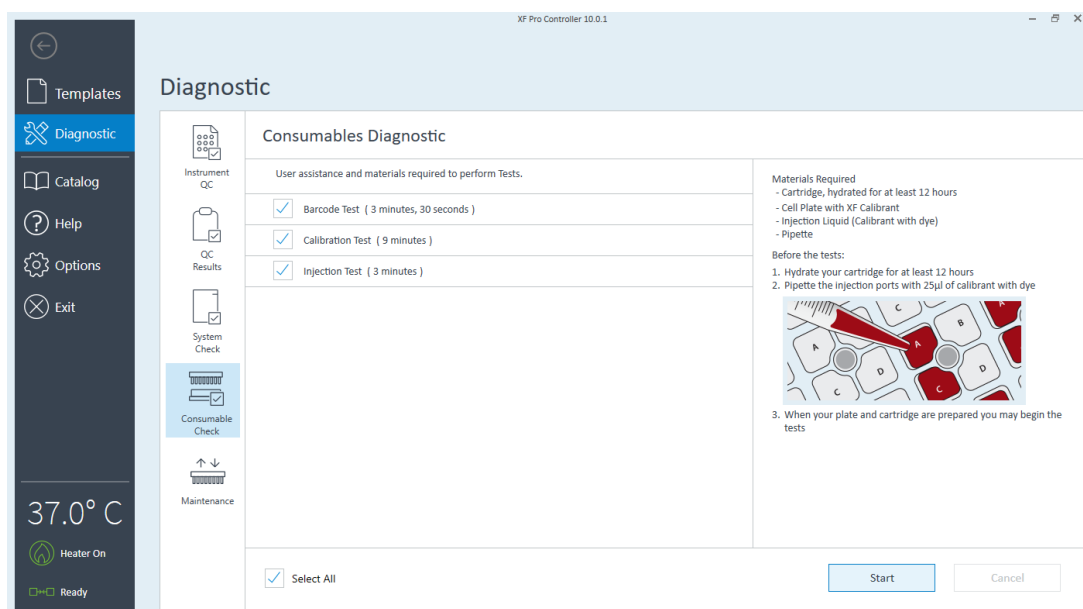


Figure 160. Diagnostic > Consumable Check view

## Maintenance

The XF Pro Analyzer is designed to require minimal cleaning and maintenance. All consumables are disposable, and none of the instrument components are exposed to the cell plate at any time, preventing cross-contamination of biological or chemical materials. However, it may be necessary to perform some tasks.

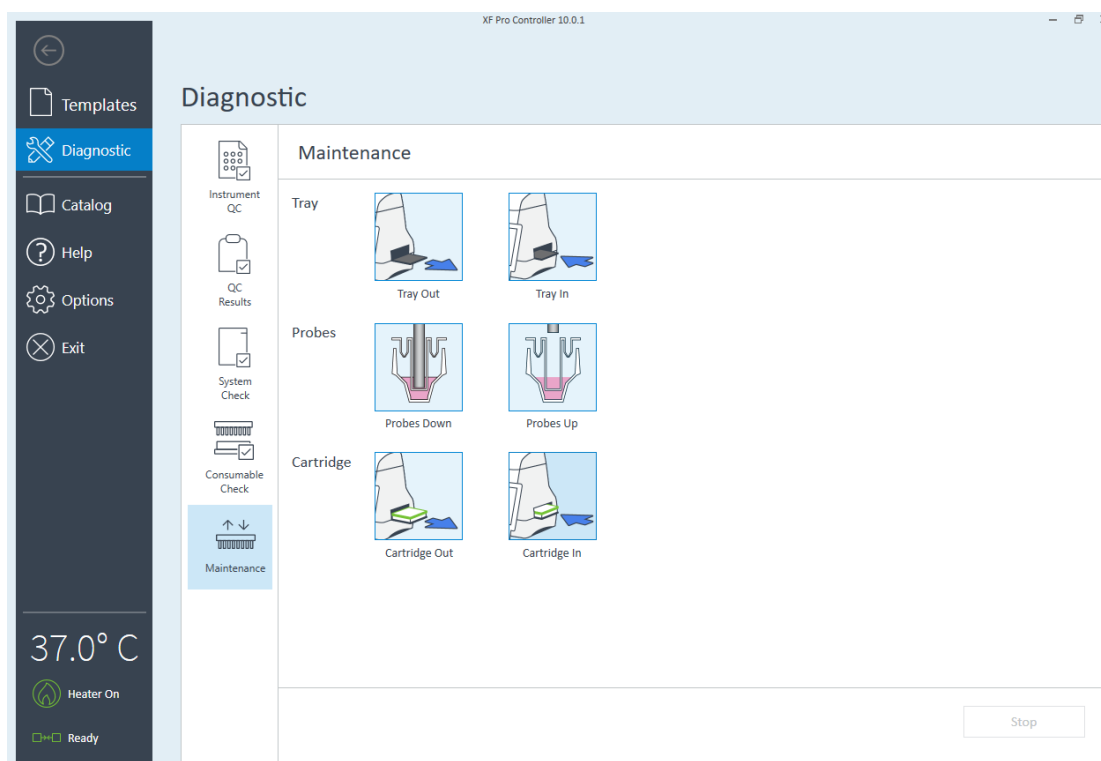


Figure 161. Diagnostic > Maintenance view

- **Tray** - Use the **Tray Control Widget** to manually eject a Utility Plate or a Cell Plate in XF Pro Analyzer. Press **Tray Out** to open the door and eject the tray. Press **Tray In** to return the tray inside the XF Pro Analyzer.
- **Probes** - Use the **Probes Widget** to load/unload a Sensor Cartridge or raise/lower probes. Probe movement will adjust the probes within the XF Pro Analyzer up or down.
- **Cartridge** - Use this function to take a Cartridge Out or put a Cartridge In the XF Pro Analyzer.

### NOTE

If a cartridge is suspected to be inside the XF Pro Analyzer, use this function to remove the cartridge before beginning another assay.

## Wave Pro & XF Pro Controller: Help view

The Help view displays software version information, Agilent Cell Analysis Technical Support contact information, and features to quickly and easily compile system files for support and diagnostic purposes.

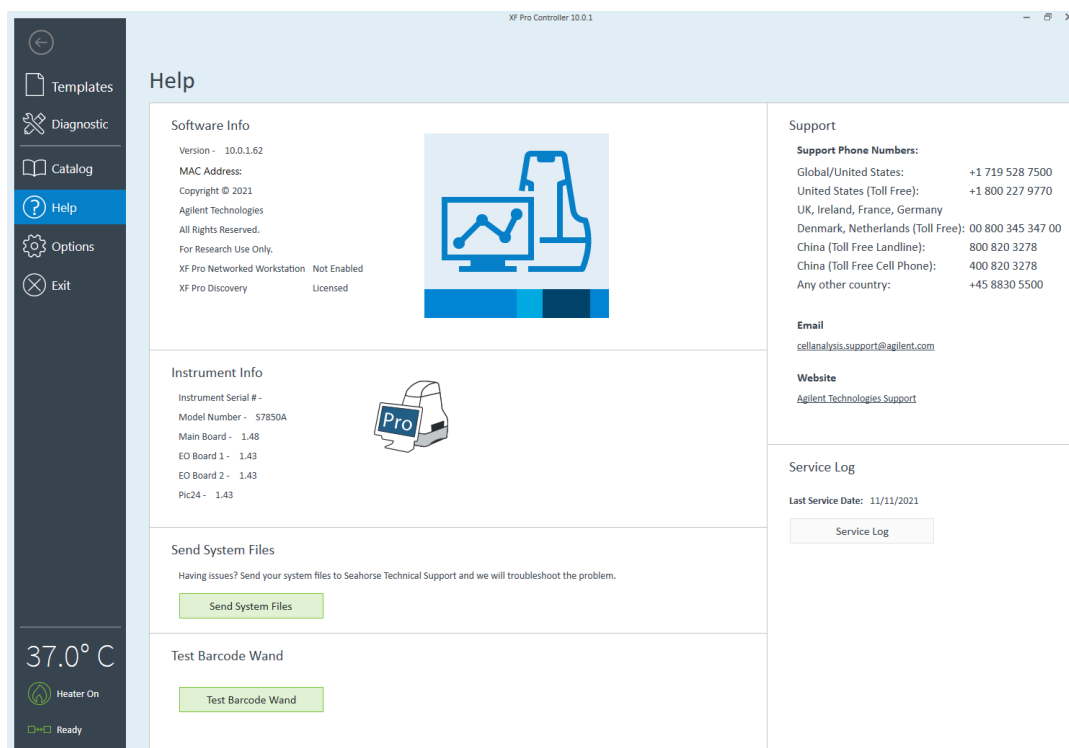


Figure 162. XF Pro Controller > Help view

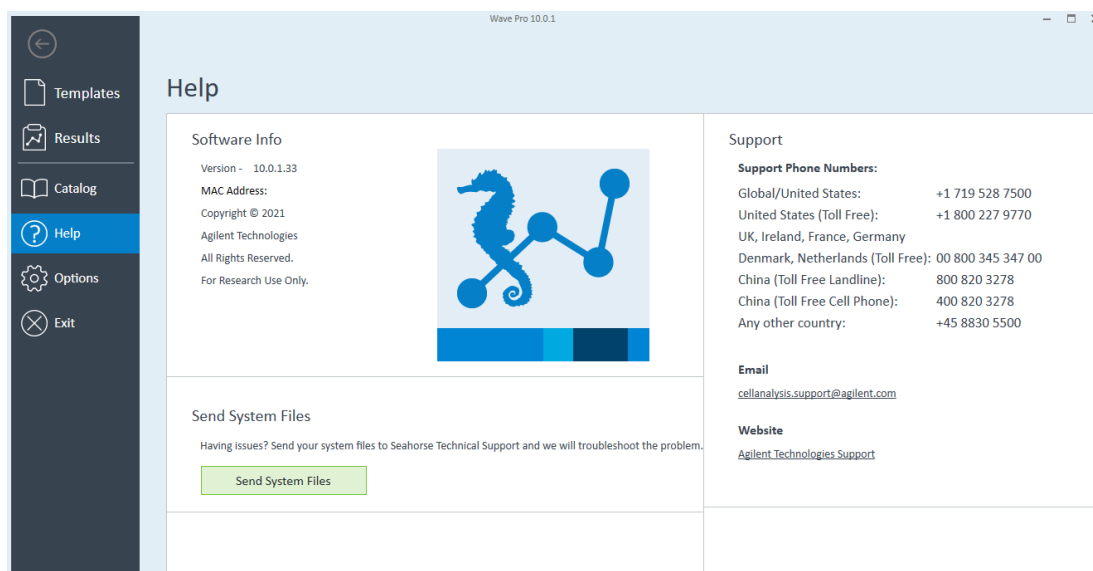


Figure 163. Wave Pro Home > Help view

## Send system files

Use the Send System Files function automatically compile system files into a compressed folder that can be attached to an email for Agilent Cell Analysis Technical Support. (See [Figure 164.](#))

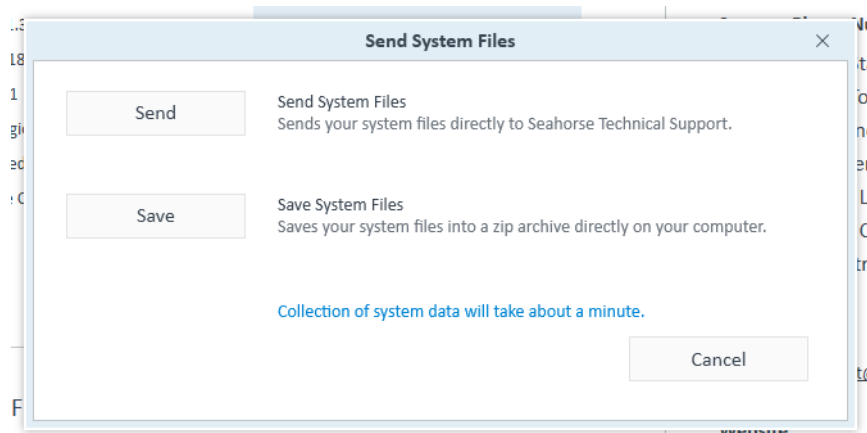


Figure 164. Send system files function

### Save system files in a compressed folder:

- 1 Open the **Help** view.
- 2 Click **Send System Files**.
- 3 Click **Save** to select a file location to save the compressed folder.

### Send system files to Agilent Cell Analysis Technical Support:

- 1 Open the **Help** view.
- 2 Click **Send**. An email message will appear populated with the following information:
  - a Email Recipient - Agilent Cell Analysis Technical Support email address.
  - b Subject - Email subject line.
  - c Email Body Text - displays the default save location of the System Files on the local drive.
- 3 Locate the saved System Files compressed folder, and attach it to the email.
- 4 Click **Send**.

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