



Agilent Seahorse XF Mito Tox Assay Kit

User Guide Kit 103595-100

For use with Agilent Seahorse XF Pro or XF Flex Analyzers.

Not recommended for use with other Agilent Seahorse XF Analyzers.

Notices

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Assay Background

The Agilent Seahorse XF Mito Tox assay is a customized workflow for use in identifying compounds exhibiting mitochondrial toxicity in drug discovery, therefore reducing risk in drug pipelines. The method developed with the kit is based on the principle that mitochondrial dysfunction due to drug-induced toxicity affects mitochondrial respiration (oxygen consumption rates or OCR, **Figure 1**). OCR-based methods (such as the Agilent Seahorse Cell Mito Stress Test) have been used previously in assessing mitochondrial toxicity and are recognized to be the most informative, sensitive, and specific methods for assessing drug-induced mitochondrial toxicity.^{1,2,3} However, the absence of a standard testing protocol or data analysis tools limits the wide adoption of oxygen consumption-based methods.

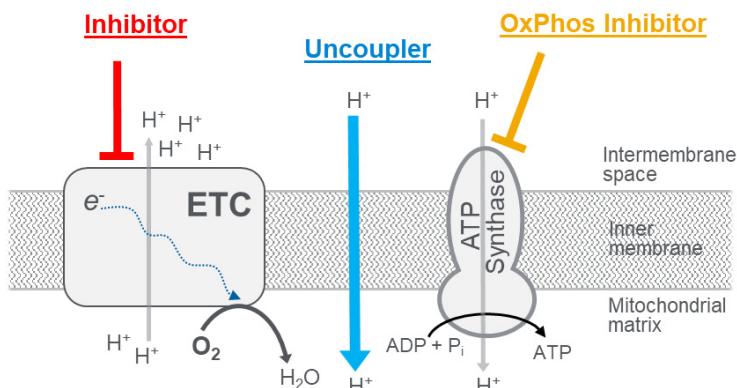


Figure 1. Modes of mitochondrial toxicity identified by the Agilent Seahorse XF Mito Tox assay. Compounds that exert effects on transport, Tricarboxylic acid (TCA) cycle, fatty acid oxidation, electron transport chain (ETC), or other upstream processes that result in decreased FCCP OCR are categorized as inhibitors. Compounds that act as protonophores, uncoupling the ETC from the OxPhos machinery and resulting in increases to Oligo OCR, are categorized as uncouplers. Compounds that specifically result in suppression of the OxPhos machinery (ATP synthase, adenine nucleotide transporter, inorganic phosphate transporter) are categorized as OPIs.

The XF Mito Tox assay is adapted from the XF Cell Mito Stress Test, the fundamental assay for interrogating mitochondrial function and dysfunction. This refined assay includes sequential injections of oligomycin and FCCP, with a separate group pre-exposed to rotenone/antimycin A (rot/AA) serving as an assay control (**Figure 2**). After completion of the assay, the kinetic OCR signals are converted to a customized parameter, the mitochondrial toxicity index (MTI), which is used to determine the mode and quantify the magnitude of mitochondrial toxicity.

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Assay Background

A test compound causing a decrease in FCCP OCR, compared to the vehicle group (MTI = 0), is classified as an inhibitor (Figure 2A). The FCCP OCR from Rot/AA group serves as a positive control for inhibition (MTI = -1). The example inhibitor in **Figure 2A** has a calculated MTI of -0.8. Conversely, a test compound eliciting an increase in Oligo OCR compared to the vehicle group (MTI = 0) is categorized as an uncoupler (Figure 2B). The FCCP OCR of the vehicle group serves as a positive control for uncoupling (MTI = 1). The example uncoupler shown in **Figure 2B** has a calculated MTI of 0.6.

In addition, a test compound causing a decrease in Basal OCR, compared to the vehicle group with little impact on FCCP OCR, is classified as an OxPhos inhibitor (Figure 2C). Note that MTI values are not calculated for OPIs.

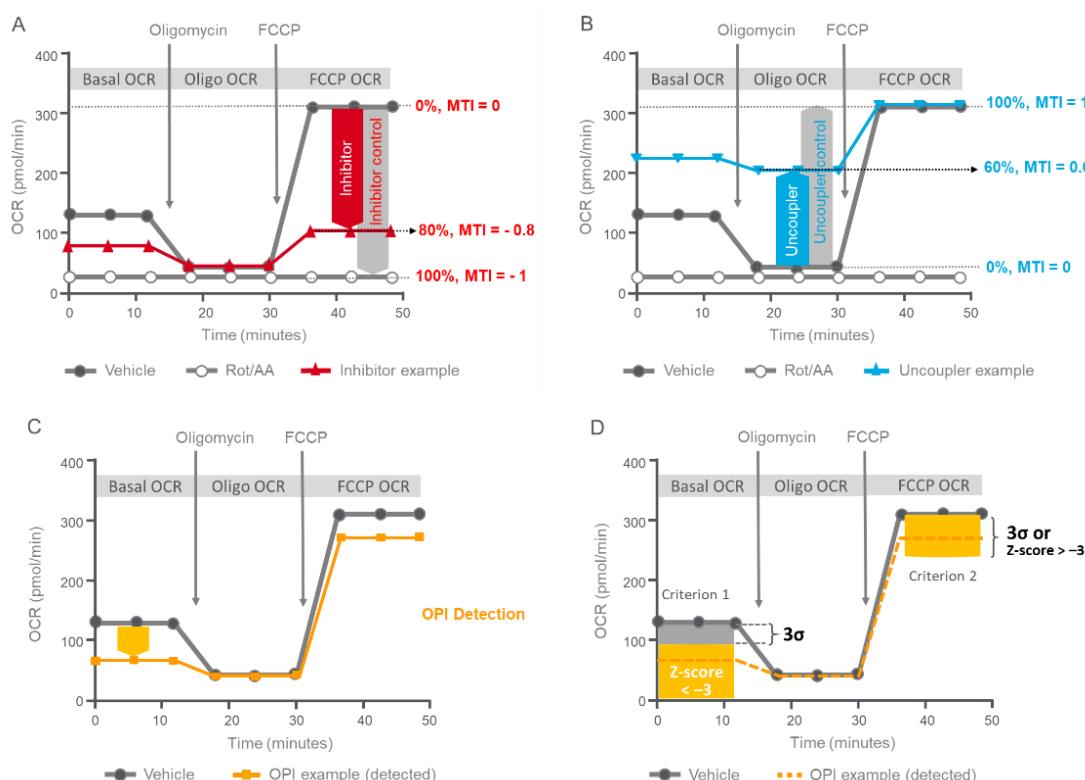


Figure 2. Seahorse XF Mito Tox assay kinetic profiles of control groups (vehicle and Rot/AA) and examples with oligomycin and FCCP injections, illustrate respiration measurements used to assess the mode and the magnitude of mitochondrial toxicity. A) Defining the MTI for inhibition. B) Defining the MTI for uncoupling. C) Defining OxPhos inhibitor (OPI). D) Criteria for OPI detection.

The assay design allows for robust assay windows to deliver assay performance ($Z'>0.5$), ensuring confident data interpretation in screening or dose-response experiments for examining mitochondrial toxicity of therapeutic compounds. For details about assay strategy, measurement definition, parameter calculation, and applications, see the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵

Figure 3 on page 7 describes three possible workflows for performing the XF Mito Tox assay using 2D cell cultures. All are identical except for the steps when the test compound is provided to the cells. It is recommended to start with workflow B (pre-incubation), where test compounds are provided to the cells when the cell culture medium is exchanged for XF assay medium. This workflow is typically the simplest to perform, while providing thorough and quantitative information about compounds eliciting mitochondrial toxicity.

1 Introduction

Glossary

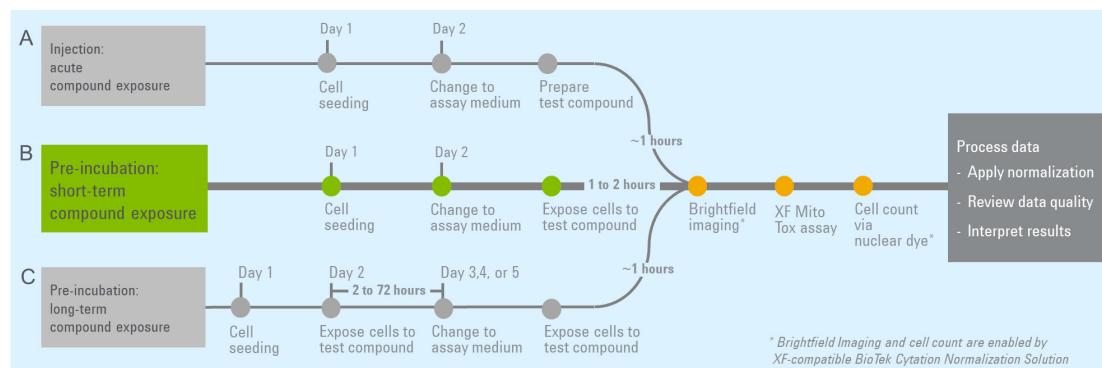


Figure 3. Streamlined workflow for the XF Mito Tox assay to investigate short- and long-term compound effects. Note that postassay cell counting for data normalization is recommended (but optional) to eliminate the impact of uneven cell plating or growth.

This user guide details how to design and perform the XF Mito Tox assay on the Seahorse XF Pro analyzer and provides initial information for data analysis.

Glossary

- **Electron transport chain (ETC):** A series of inner mitochondrial membrane protein complexes (including CI to CIV) that transfer electrons from electron donors to electron acceptors via redox reactions. The energy from these redox reactions creates an electrochemical proton gradient that drives the synthesis of adenosine triphosphate (ATP) via the OxPhos machinery.
- **OxPhos machinery:** Mitochondrial protein complexes involved in adenosine triphosphate (ATP) production, including the ATP synthase (Complex V), adenine nucleotide transporter (ANT), and inorganic phosphate transporter (P_iT). The OxPhos machinery is coupled to the ETC via the proton gradient that drives the ATP synthase.
- **OCR:** Oxygen consumption rate
- **Basal OCR:** Oxygen consumption rate measured before oligomycin injection.
- **Oligo OCR:** Oxygen consumption rate measured after oligomycin injection, but before FCCP injection.
- **FCCP OCR:** Oxygen consumption rate measured after FCCP injection.
- **Vehicle control group:** A group of wells that is treated with the same final concentration of solvent present when using test compounds (for example, DMSO).
- **Rot/AA control group:** A group of wells that is provided with rotenone/antimycin A in the pretreatment step before the assay.
- **Mitochondrial Toxicity Index (MTI):** A parameter used to identify and quantitate mitochondrial toxicity. Positive MTI values (typically between 0 and 1) identify mitochondrial toxicity due to uncoupling. Negative MTI values (typically between 0 and -1) identify mitochondrial toxicity due to inhibition. See the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵ for further information on MTI.
- **Inhibitor:** A compound that decreases FCCP OCR compared to maximal FCCP OCR of the vehicle group, with typical MTI values ranging from 0 to -1.

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- **Uncoupler:** A compound that increases Oligo OCR compared to the minimal Oligo OCR of the vehicle control group, with typical MTI values ranging from 0 to 1.
- **OxPhos inhibitor (OPI):** A compound that decreases Basal OCR significantly, compared to Basal OCR of the vehicle control group, but does not significantly impact FCCP OCR compared to FCCP OCR of the vehicle control group.
- **Short-term XF Mito Tox assay workflow:** Used to assess short-term effects of test compounds on mitochondrial function. Test compounds are provided to the cells 1 to 2 hours before performing the XF assay. This is the recommended standard protocol.
- **Acute XF Mito Tox assay workflow:** Used to assess any real-time changes in mitochondrial function within minutes of test compound exposure. Test compounds are provided to the cells via injection port A after Basal OCR measurements, but before oligomycin injection.
- **Long-term XF Mito Tox assay workflow:** Used to assess the long-term effects of test compounds on mitochondrial function. Test compounds are usually incubated with cells 4 to 72 hours before performing the XF assay.
- **Acute response:** A change in OCR due to an injection of test compound after Basal OCR measurements and before oligomycin injection.
- **Z' factor (Z'):** Z' is used as a measure of assay quality or assay performance and is influenced by dynamic range and standard error in the resulting assay data. Z' factors are provided for both uncoupling and inhibition results to allow assessment of assay performance. See the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵ for further information on Z'.

References

- 1 Will *et al.* *Nat Protoc.* **2006**, 1(6), 2563–2572. DOI: [10.1038/nprot.2006.351](https://doi.org/10.1038/nprot.2006.351).
- 2 Will Y and Dykens J. *Expert Opin Drug Metab Toxicol.* **2014**, 10:8, 1061. 1067 DOI:[10.1517/17425255.2014.939628](https://doi.org/10.1517/17425255.2014.939628).
- 3 Tilmant *et al.* *Toxicol in Vitro.* **2018**, 52, 374–383. DOI:[10.1016/j.tiv.2018.07.012](https://doi.org/10.1016/j.tiv.2018.07.012).
- 4 Rogers *et al.* Principle of Mitochondrial Toxicity Assessment Using Agilent Seahorse XF Solution *Agilent Technologies White Paper*, publication number 5994-4732EN.
- 5 Kam *et al.* A Customized XF Workflow for Detection and Characterization of Mitochondrial Toxicity *Agilent Technologies Application Note*, publication number 5994-4778EN.

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Kit Contents

The Agilent Seahorse XF Mito Tox Assay kit includes six foil pouches, and each contains reagents sufficient for a full-plate assay in a 96-well format. Every pouch includes one vial of the following compounds: oligomycin, FCCP, and a mixture of rotenone/antimycin A (see **Table 1**).

Table 1 **Seahorse XF Mito Tox Assay Kit contents.**

| Compound | Cap Color | Quantity |
|-------------------------------|-----------|---------------|
| Oligomycin | Blue | 63 nmol |
| FCCP | Yellow | 72 nmol |
| Rotenone/Antimycin A (Rot/AA) | Red | 5.4 nmol each |

Kit Shipping and Storage

The Agilent Seahorse XF Mito Tox assay is shipped and stored at room temperature. The product is stable for one year from the date of manufacture. The actual expiration date is printed on the label of the assay kit box. Depending on the shipping date, the actual shelf life of the kit in the user's hand can vary between 3 and 12 months.

2 Kit Information

Additional Required Items

Additional Required Items

The Agilent products in **Table 2** are required for performing Seahorse XF Mito Tox assays, but are not supplied with the kit. For a complete list of materials required to perform an XF assay, please visit the [Agilent Cell Analysis Learning Center](#) website.

Table 2 Additional required items.

| Item | Part Number |
|---|-----------------------------|
| Agilent Seahorse XF Pro analyzer | |
| Agilent Seahorse XF Pro M FluxPak, 18 assays* | 103775-100 |
| Agilent Seahorse XF Pro M FluxPak, 6 assays* | 103777-100 |
| Agilent Seahorse XF Pro M Cell Culture Plate, 6 plates [†] | 103774-100 |
| Agilent Seahorse XF DMEM Medium pH 7.4 [‡] | 103575-100 |
| Agilent Seahorse XF 1.0 M Glucose solution | 103577-100 |
| Agilent Seahorse XF 100 mM Pyruvate solution | 103578-100 |
| Agilent Seahorse XF 200 mM Glutamine solution | 103579-100 |
| Agilent Reservoir, 12 column, polypropylene or Agilent Reservoir, 12 column, polypropylene, irradiated | 201280-100 or 204365-100 |

* Part numbers 103775-100 and 103777-100 contain Agilent Seahorse XF sensor cartridges and XF Pro M cell culture plates.

† Part number 103774-100 does not contain XF sensor cartridges, which are also required to perform XF assays. This part number is only needed when extra plates are required for optimizing seeding density.

‡ XF DMEM medium can also be purchased together with the supplements listed in this table as bundled products (part number 103680-100).

Pre-assay Optimization Requirements

The optimal compound concentration for obtaining robust results is cell line dependent. It is therefore recommended that a titration experiment for the compounds provided in the kit is performed for each new cell type before performing the XF Mito Tox assay. Some assay conditions can also affect optimal compound concentration, such as inclusion of BSA or serum in assay medium. In these cases, an optimization experiment is also needed.

This is critically important for FCCP. FCCP is a tool chemical commonly used to estimate the maximal respiratory capacity in cells. Suboptimal concentration will result in a reduced assay window and therefore reduced assay performance. Before performing XF Mito Tox assays, an FCCP optimization experiment must be carried out whenever a new cell type or some new assay conditions are used. The FCCP optimization experiments can be performed using the Agilent Seahorse XF Cell Mito Stress Test Kit. A standard protocol is provided in the document: *FCCP Optimization Using the Agilent Seahorse XF Cell Mito Stress Test with the Agilent Seahorse XF Pro Analyzer*.

For oligomycin, 1.5 μ M is recommended, while for Rot/AA, 0.5 μ M is recommended, for most cell types. For assistance with any further questions, please contact Agilent **Cell Analysis Technical Support**.

Cell seeding density must also be optimized for the cell type used. Cell seeding density optimization can be combined with the FCCP optimization experiment. For HepG2 cells, the optimal seeding density is around 20×10^3 cells/well in XF Pro M cell culture plates. When beginning to work with HepG2 cells, testing three seeding densities of 10×10^3 , 20×10^3 , and 30×10^3 cells/well is recommended to finalize the optimal seeding density. In general, the FCCP stimulated OCR should not exceed 350 pmoles/min. If it does, seeding density should be decreased.

Before performing compound testing or screening, assay performance (Z') assessment is encouraged during assay validation. Further details of the Z' calculation and examples can be found in the Agilent white paper by Roger *et al.*⁴ and the Agilent application note by Kam *et al.*⁵

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Assay Template Design and Creation

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This section describes the procedures for creating XF Mito Tox assay templates.

An XF assay template provides the instrument protocol that programs the Agilent Seahorse XF Pro analyzer to collect measurements and execute injections, and must be created before performing the assay. The assay design information in the template is also required to support downstream data transformation within Agilent Seahorse Analytics.

For the XF Mito Tox assay, templates are created from the **blank** template. Either the Agilent Seahorse XF Pro Controller or Agilent Seahorse XF Wave Pro software installed on a desktop computer can be used to design the assay template.

4 Assay Template Design and Creation

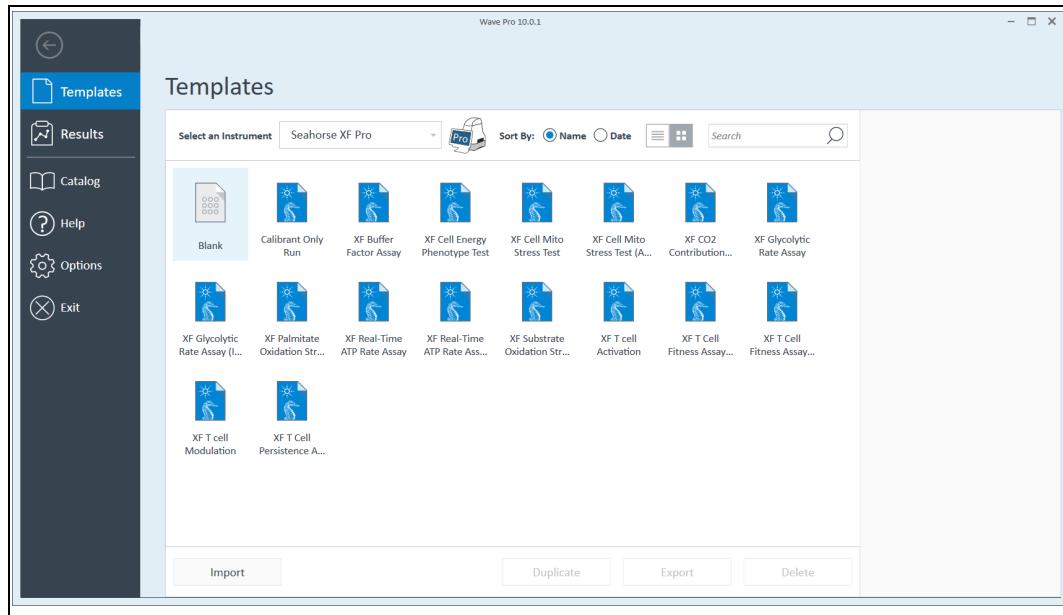
Template Creation for Testing a Small Set of Compounds

Template Creation for Testing a Small Set of Compounds

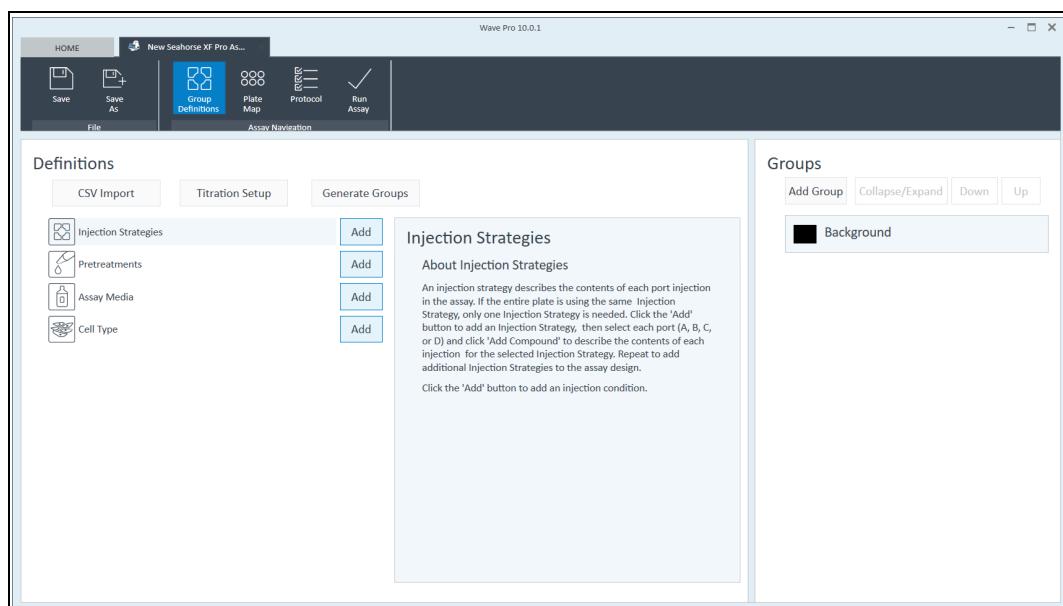
The procedure described in this section is intended for experiments that test a small set of compounds (for example, 10 test compounds per plate at a single dose).

Define injection strategies, assay media and cell types

- 1 Open Wave Pro software. Double click the blank template.



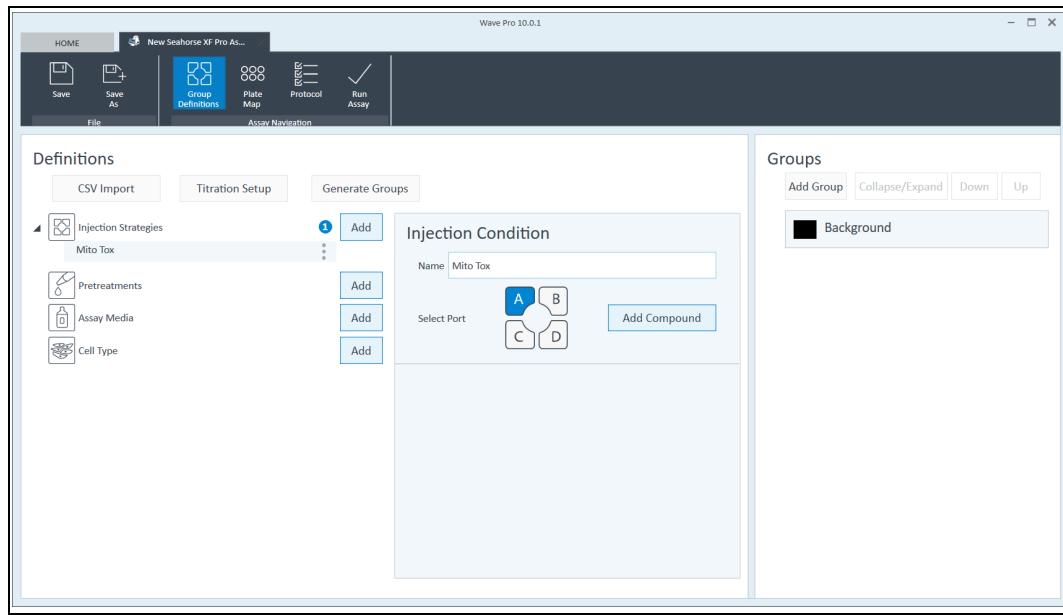
- 2 The window below will appear. The description in the middle panel explains what an injection strategy is and how to add one.



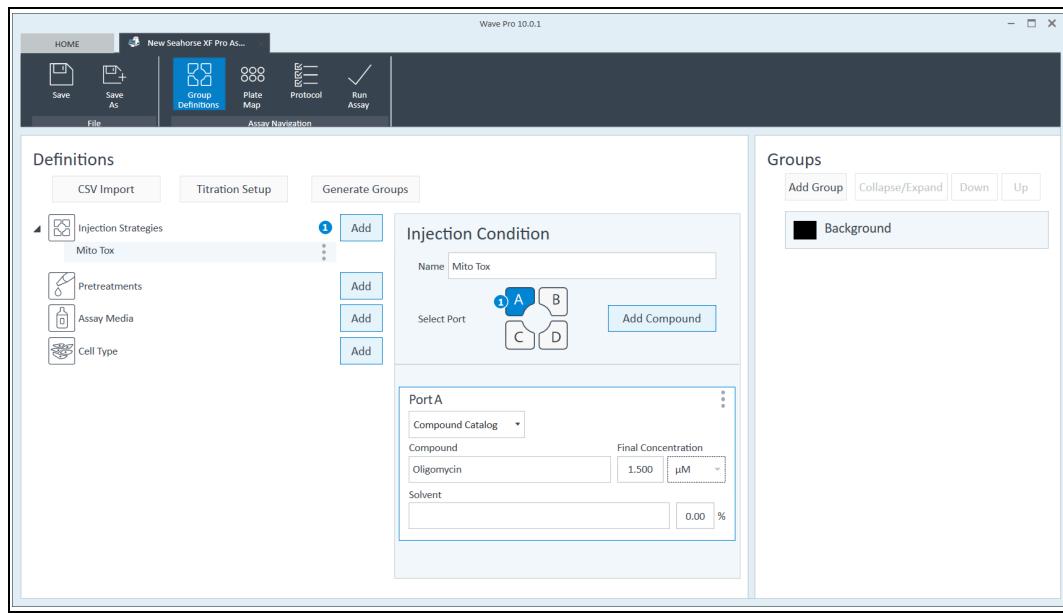
4 Assay Template Design and Creation

Define injection strategies, assay media and cell types

- 3 Click **Add** on the right side of the **Injection Strategies** box. The following information will appear in the middle panel.

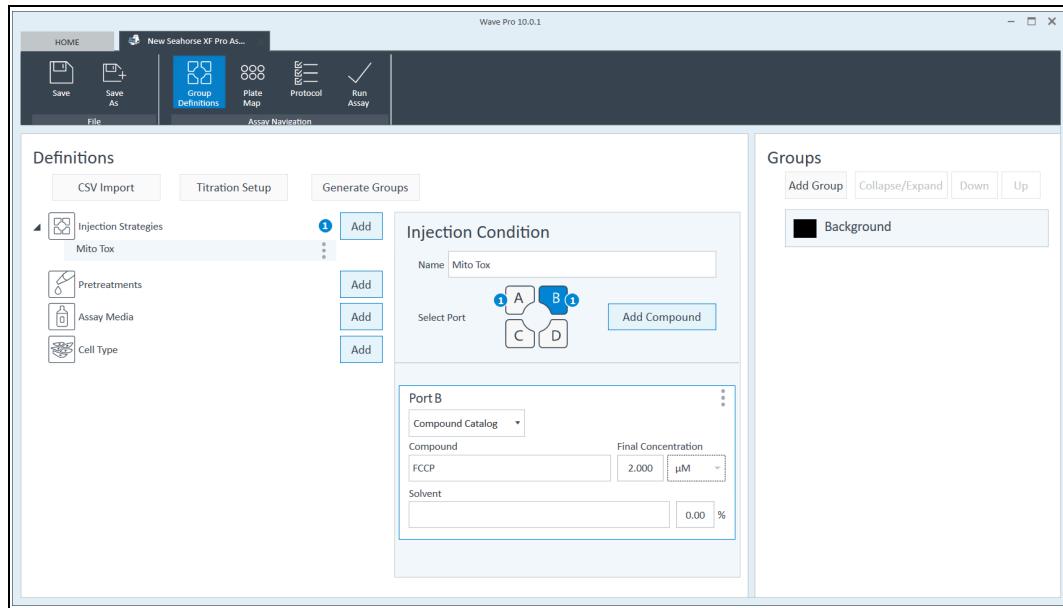


- 4 Type "Mito Tox" in the **Name** box. To assign injection compounds to each port, select the port first, then click **Add Compound**. For Port A, enter oligomycin and 1.5 μ M for the final concentration. For Port B, enter FCCP and the final concentration (optimal final concentration predetermined, see "[Pre-assay Optimization Requirements](#)" on page 11).

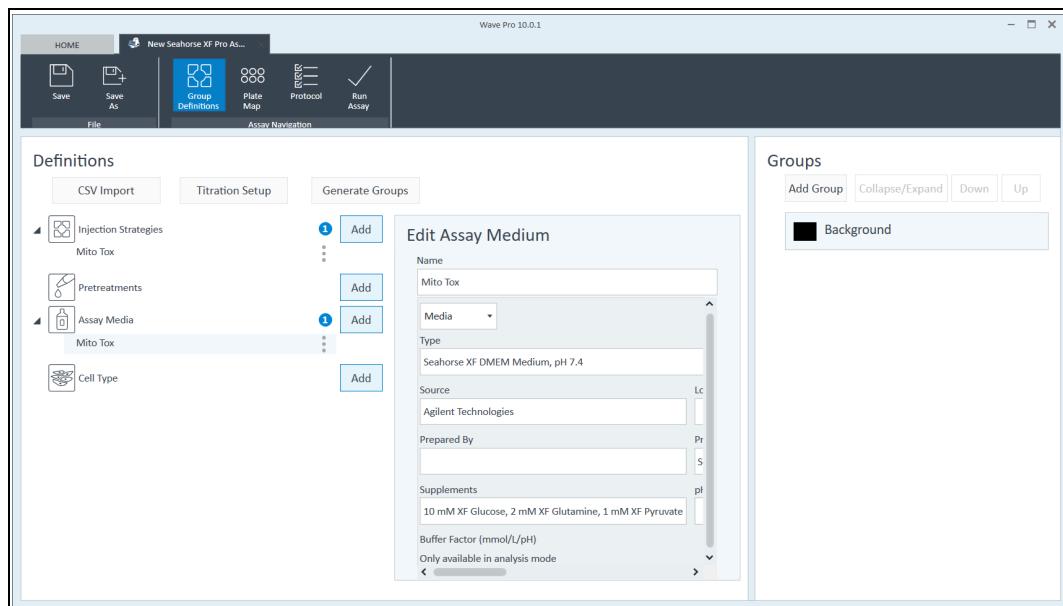


4 Assay Template Design and Creation

Define injection strategies, assay media and cell types



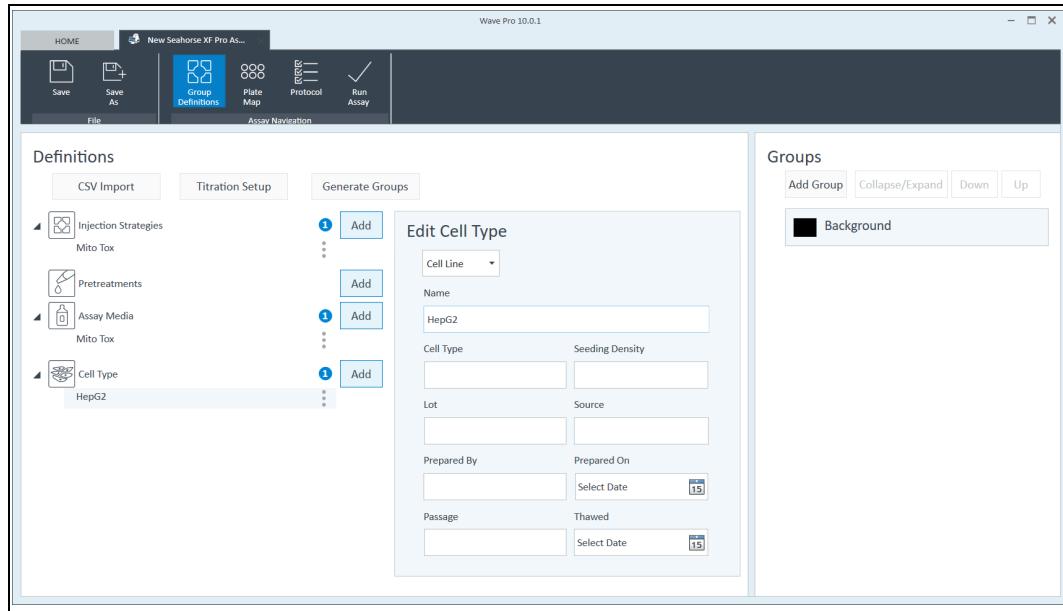
- 5 Next, define the assay medium. Click **Add** to the right of **Assay Media** box. Enter the name and select the assay medium type from the **Media** drop-down menu (for example, XF DMEM pH 7.4, supplemented with 10 mM XF Glucose, 1 mM XF Pyruvate, and 2 mM XF Glutamine).



4 Assay Template Design and Creation

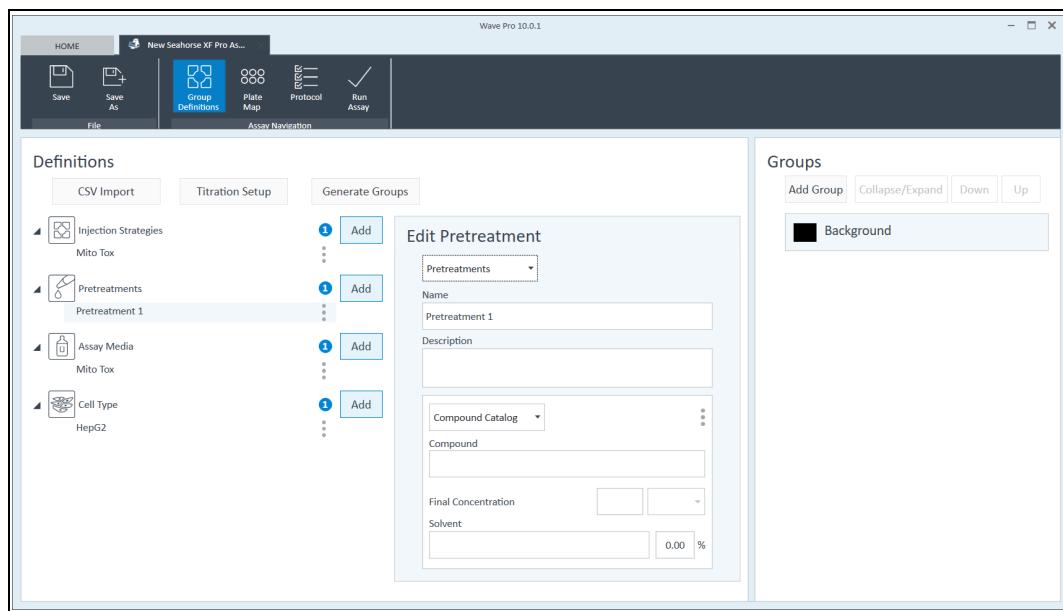
Define experimental groups and plate layout

- 6 To define **Cell Type**, click **Add** to the right of the **Cell Type** box, and enter the details about the cell type being assayed.



Define experimental groups and plate layout

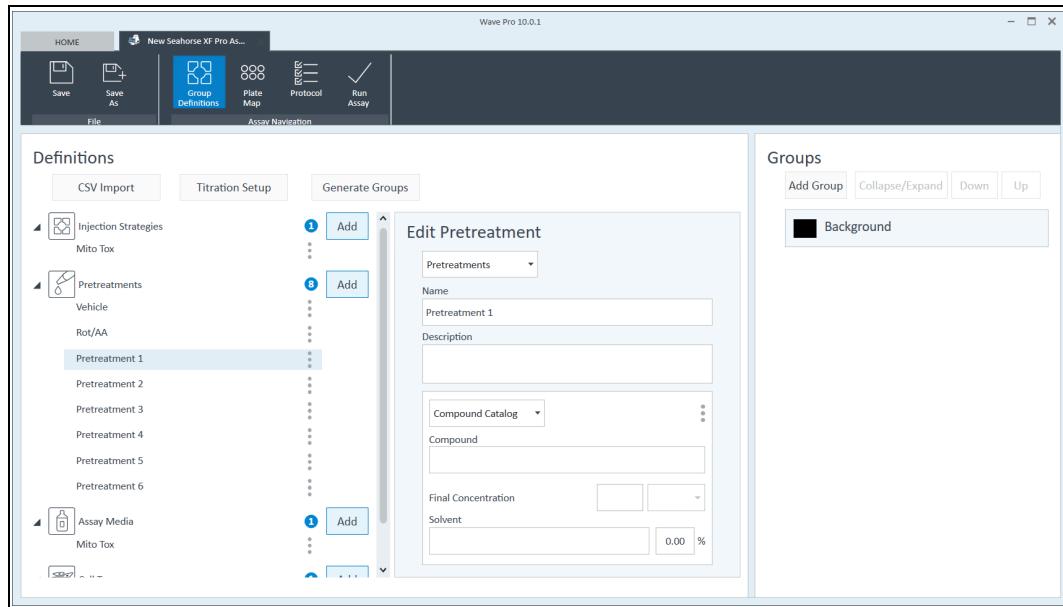
- 1 First, define pretreatments/conditions for experimental groups and click **Add** to the right of **Pretreatments** box.



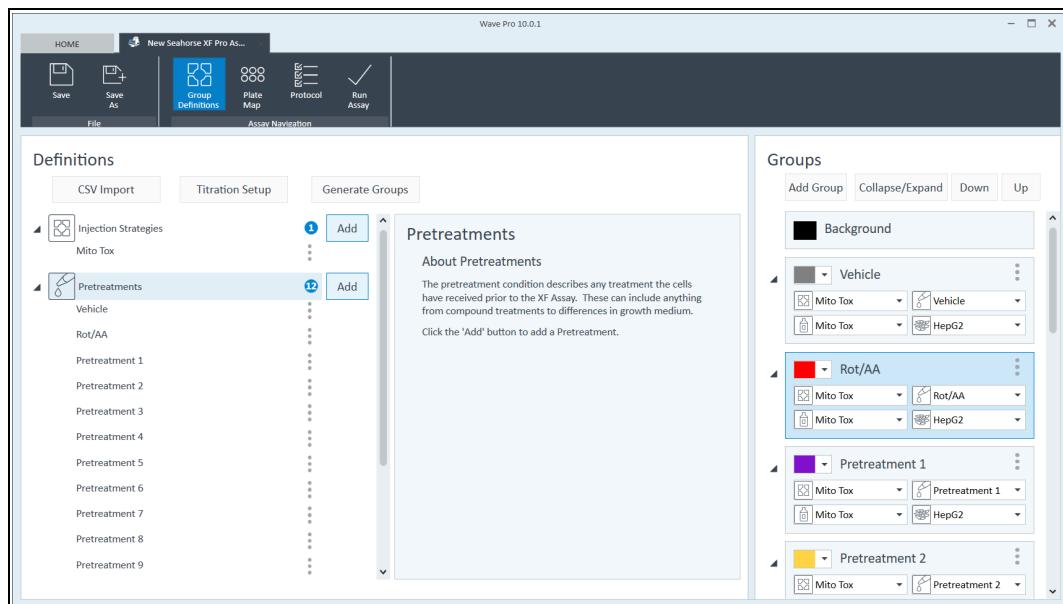
4 Assay Template Design and Creation

Define experimental groups and plate layout

- 2 Use this function to create pretreatments/conditions for two control groups: the vehicle group and the Rot/AA group, and any additional experimental groups treated with test compounds. Treatment names can be manually changed in the editing view in the middle panel. A treatment can be deleted by clicking the three-dot menu to the right of each treatment.



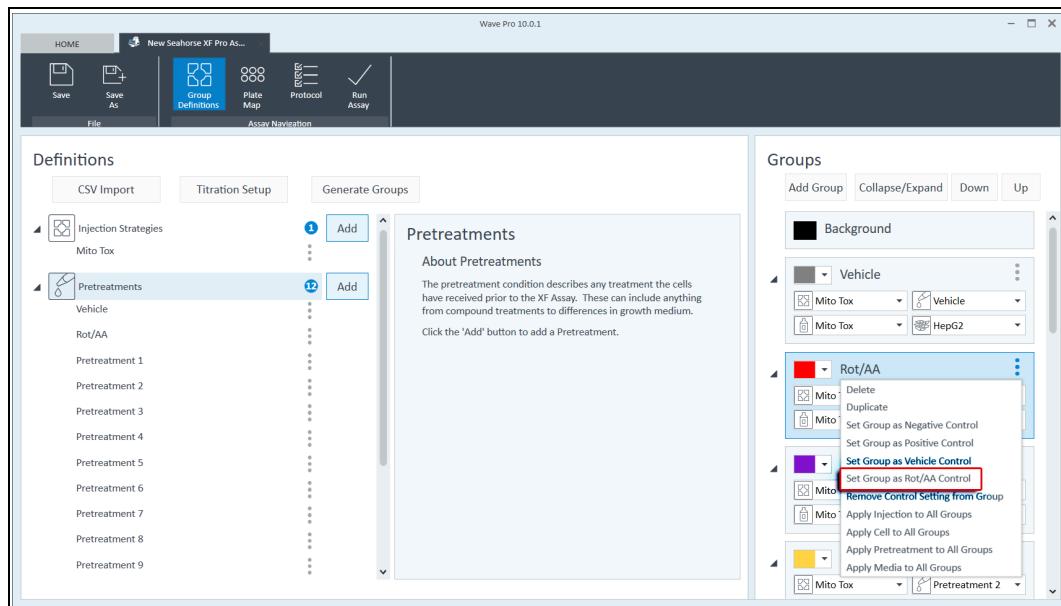
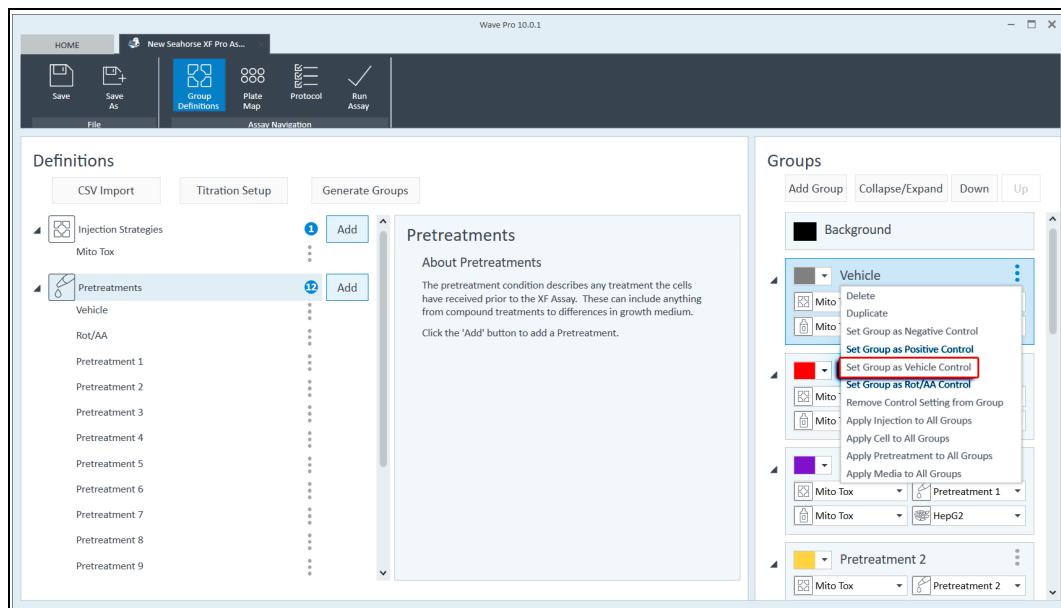
- 3 After all treatment/conditions are created, click **Generate Groups** at the top of the window, under **Definitions** (next to **Titration Setup**). Groups are generated, one for each of the pretreatments/conditions. The predefined injection strategy, media cell type, and pretreatment are assigned automatically to the groups generated (see the right side panel in the screenshot below).



4 Assay Template Design and Creation

Define experimental groups and plate layout

- 4 Click the three-dot menu next to vehicle group and select **Set Group as Vehicle Control**. Change the color to gray if desired. Repeat this step for the Rot/AA group to select **Set Group as Rot/AA Control**, and change the color to red if desired.



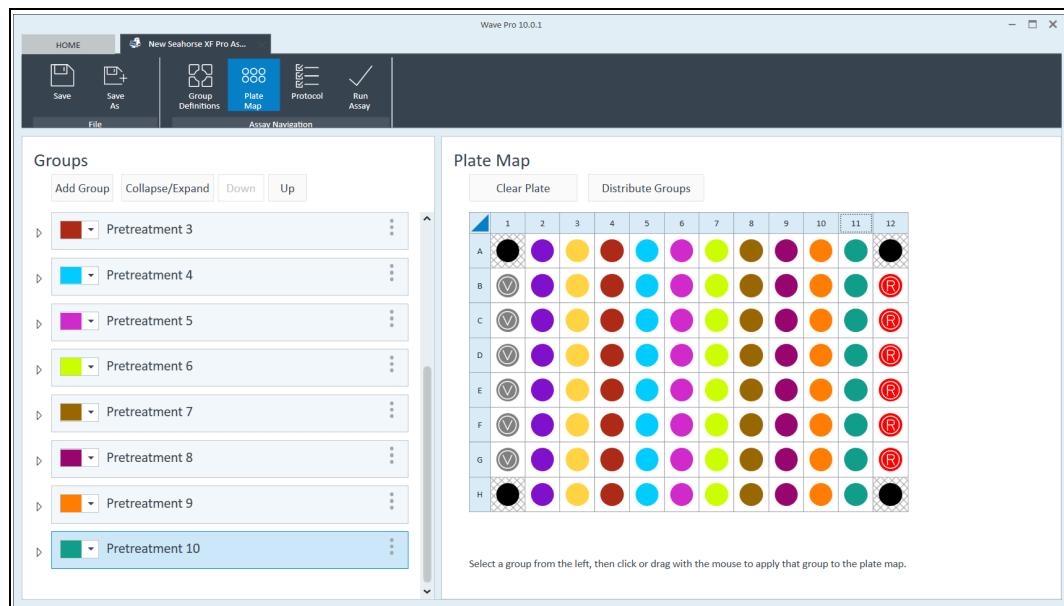
4 Assay Template Design and Creation

Define experimental groups and plate layout

- 5 Next, switch to the **Plate Map** tab and assign groups to the desired wells. First select/highlight the specific group (under **Groups**) and then click or drag the mouse across the wells to be assigned in the **Plate Map**.

NOTE

Placing vehicle in column 1 (row B to G) and Rot/AA in column 12 (row B to G) is recommended. Ensure that the controls are assigned properly. A "V" will be displayed in the vehicle wells and an "R" will be displayed in the Rot/AA wells. The MTI parameter cannot be calculated without these groups.

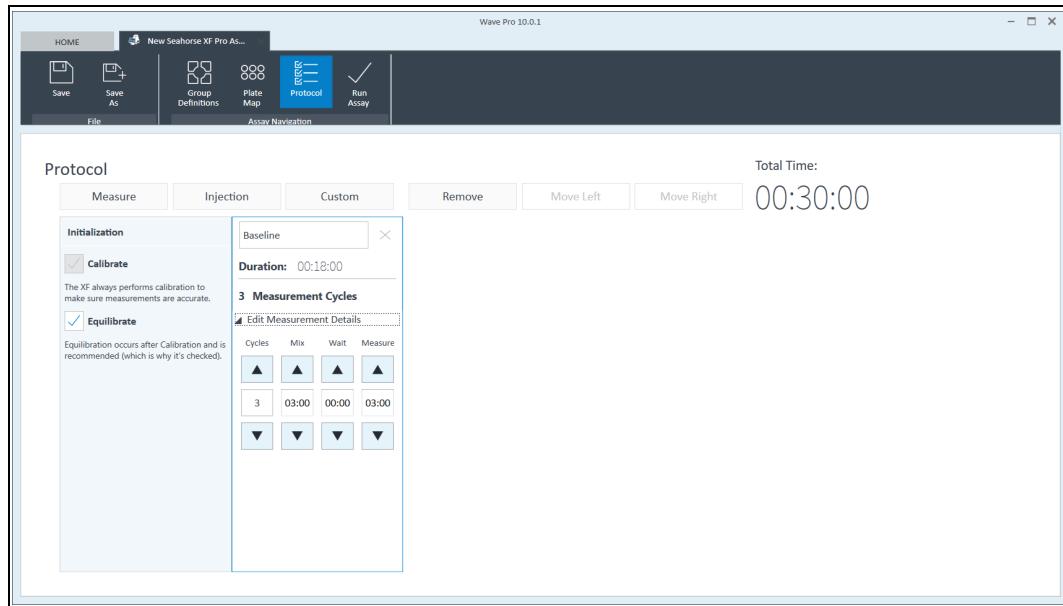


4 Assay Template Design and Creation

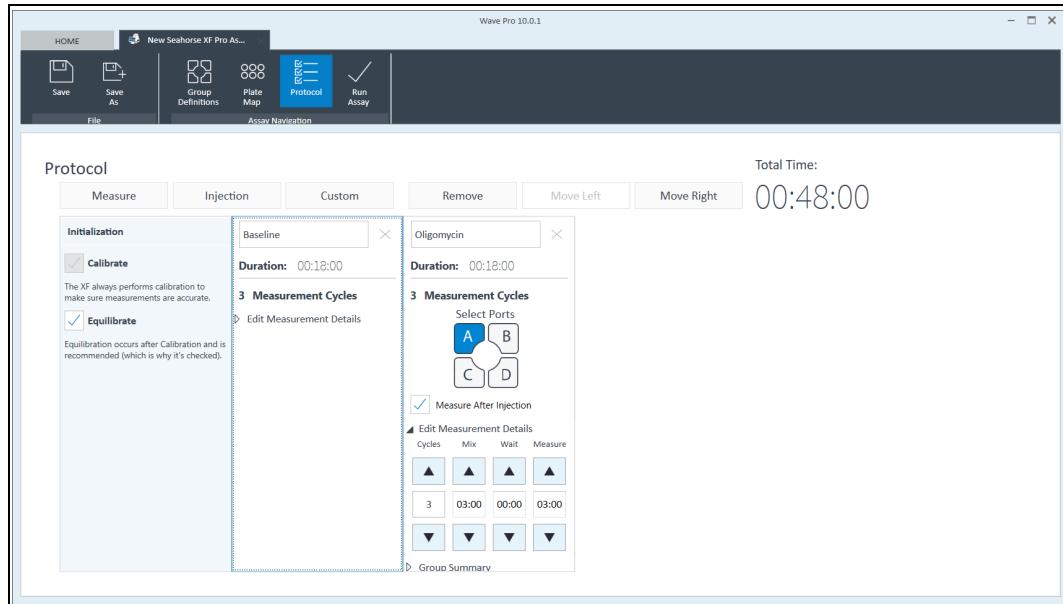
Define instrument protocol

Define instrument protocol

- 1 Switch to the **Protocol** tab. The blank template is built with a **Baseline** step, which includes three basal measurements.



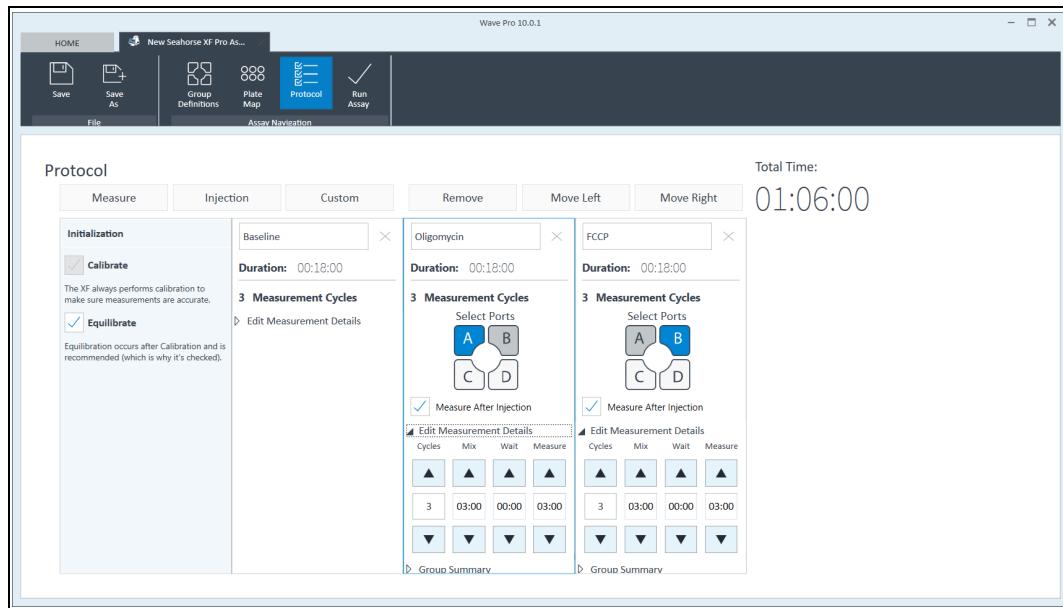
- 2 Next, add an injection step by clicking the **Injection** box. Enter oligomycin to replace "injection 1". Ensure Port A is highlighted; this indicates that oligomycin will be injected through Port A. This step includes three measurements following the injection. Changing the measurement time and numbers for performing the XF Mito Tox assays is not recommended, but can be done if necessary.



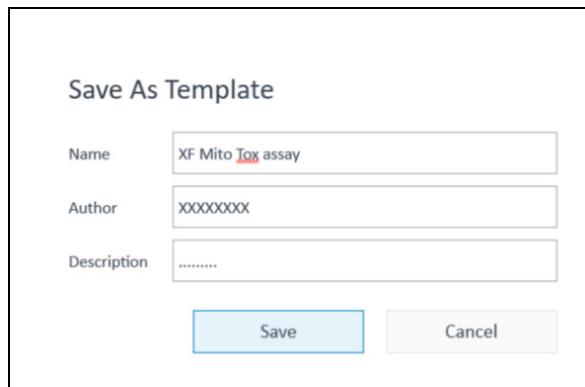
4 Assay Template Design and Creation

Define instrument protocol

- 3 Add another Injection step by clicking the **Injection** box again. Enter FCCP to replace "injection 2". Ensure Port B is highlighted, which indicates that FCCP will be injected through Port B. This step also includes three measurements following the injection.



- 4 The template creation is now complete. Click the **Save** symbol at the top left, name the template, and click **Save** in the dialog box.



NOTE

A template for Z' assessment can be created following the instructions provided in this section. For more information about Z' assessment assay, please refer to Agilent white paper by Rogers et al⁴ and the Agilent application note by Kam et al.⁵

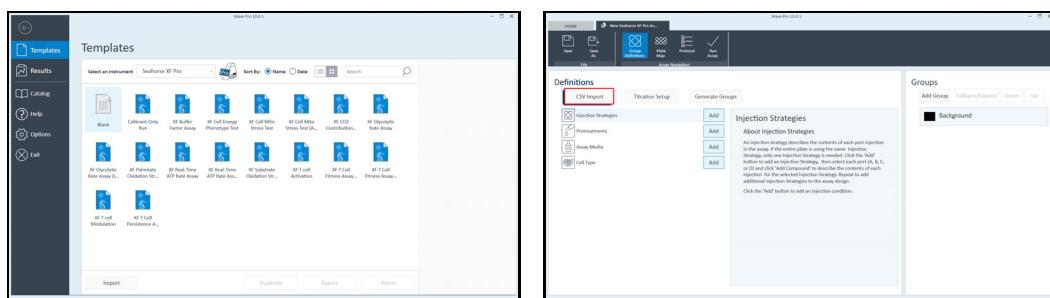
4 Assay Template Design and Creation

Template Creation for Testing a Large Set of Compounds

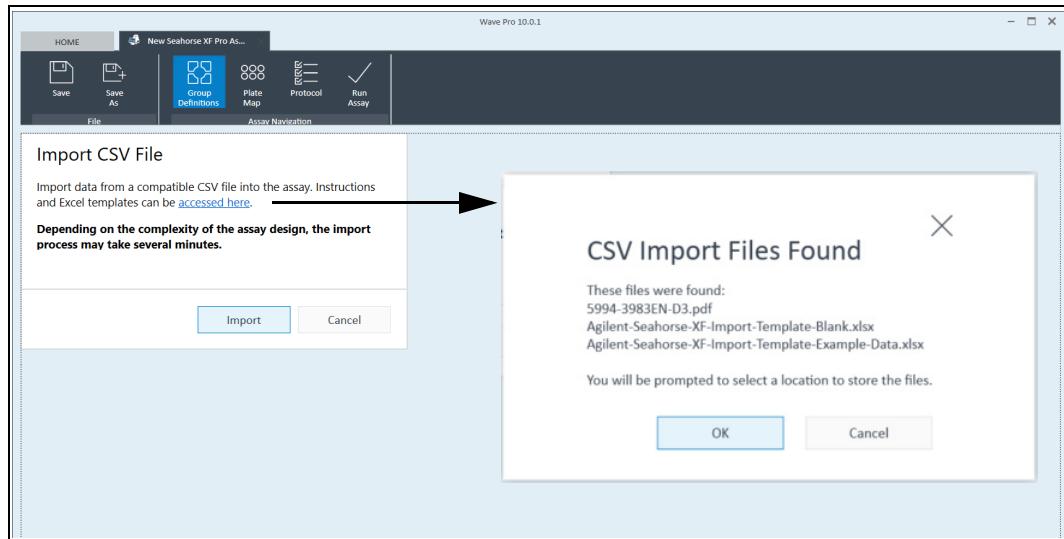
Template Creation for Testing a Large Set of Compounds

The procedure described in this section is intended for screening experiments that test large set of compounds (for example, 80 test compounds per plate at single dose). Here, the CSV import function can be used to minimize the time spent creating an assay template. The design attributes in the CSV file include group definition, injection strategy, assay media, and cell type, as well as plate map.

- 1 Open the Wave Pro software. Double click the blank template. In the new view, Select **CSV Import**.



- 2 In the view shown below, click the link (accessed here) to download the Excel template file. Click **OK** in the pop-up dialog box. A detailed set of instructions on how to use this feature and how to fill the template, as well as an example file, will be downloaded automatically.

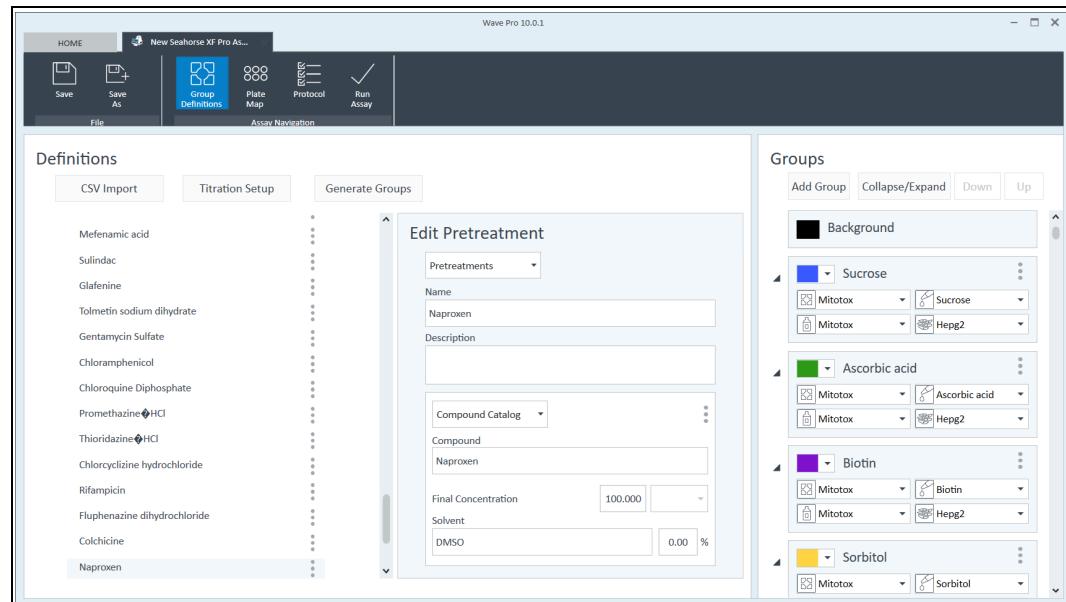


4 Assay Template Design and Creation

Template Creation for Testing a Large Set of Compounds

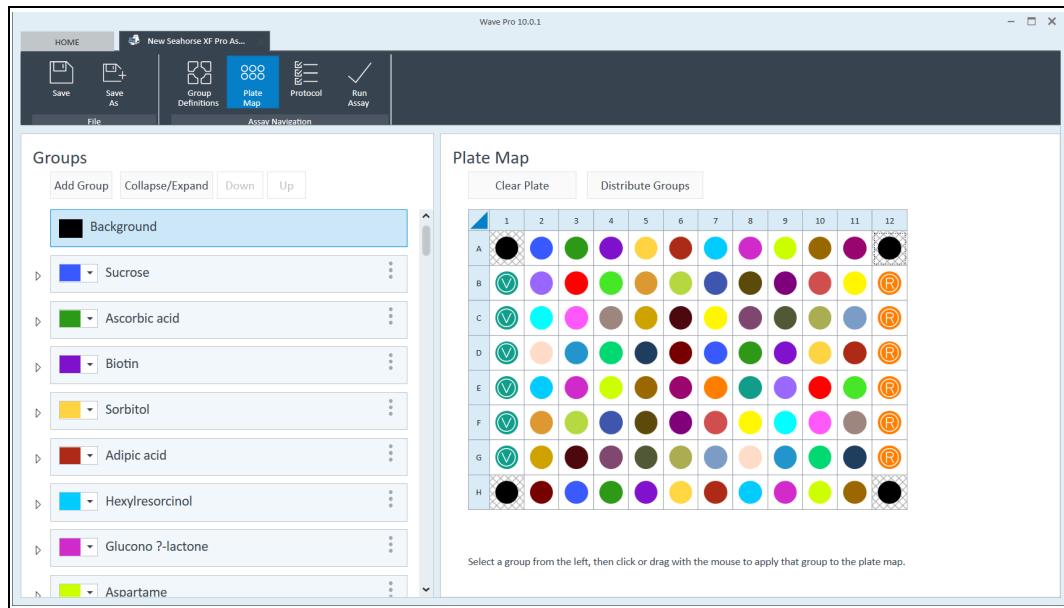
- 3 Follow the instructions in the downloaded file to complete the template. The screenshot below is an example of completed CSV template for a screening of 80 compounds.

- 4 After filling out the required information, save the template in a .CSV format. Close the file and Excel program. Return to the Wave Pro software.
- 5 Select the **Import** option to load the CSV file into Wave Pro. Browse to find the template and open it. It may take several minutes to complete the import.
- 6 After the import is finished, the **Group Definition** view will appear. Review all groups, including the vehicle and Rot/AA controls, to ensure that these groups have been assigned properly. If not, scroll through the group list and assign the controls.

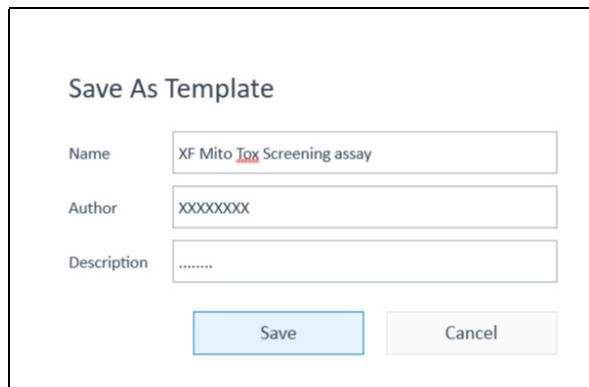


4 Assay Template Design and Creation

Template Creation for Testing a Large Set of Compounds



- 7 Switch to the **Protocol** tab. Follow the steps described in the section **“Template Creation for Testing a Small Set of Compounds”** on page 14 to define the instrument protocol.
- 8 The template creation is now complete. Click the **Save** symbol at the top left, name the template, and click **Save** in the dialog box.



Template Creation for Dose-Response Assays

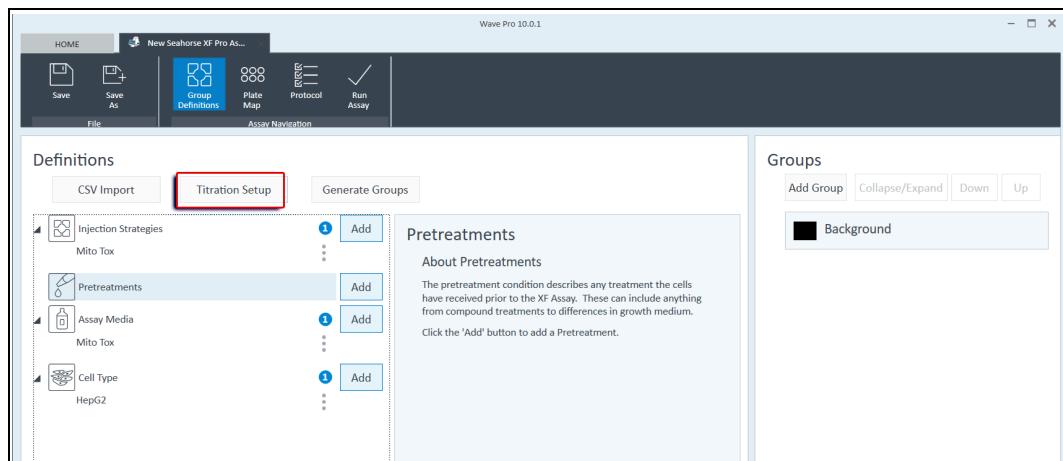
The procedure described in this section is intended for performing dose-response assays (for example, eight test compounds per plate, 10 doses per compound).

Define injection strategies, assay media and cell types

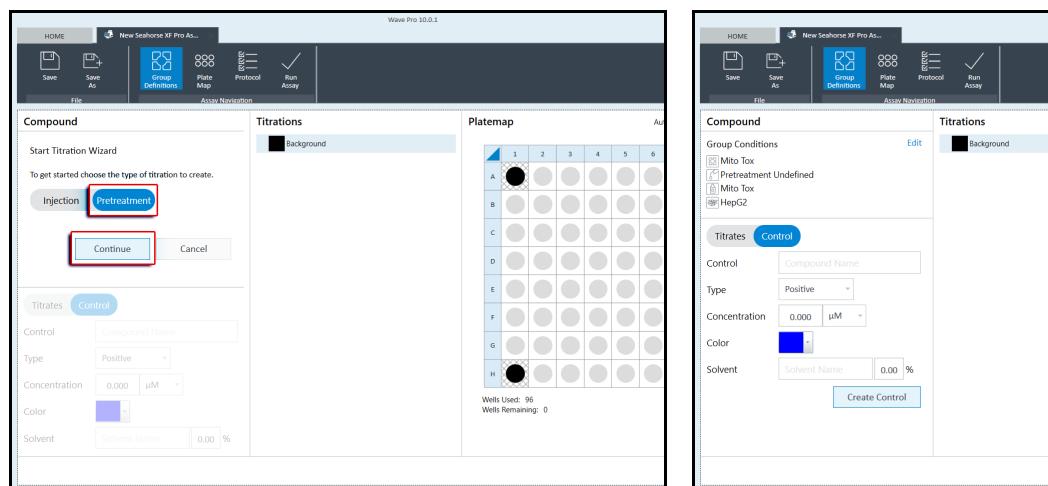
Follow the steps described in the section “[Template Creation for Testing a Small Set of Compounds](#)” on page 14 to define injection strategies, assay media, and cell types, as the procedure is the same. These attributes should be defined first, as they will be automatically assigned to all titrations and control groups when **Titration Setup** is used.

Define experimental groups and plate layout

- 1 Select the **Titration Setup** tab.



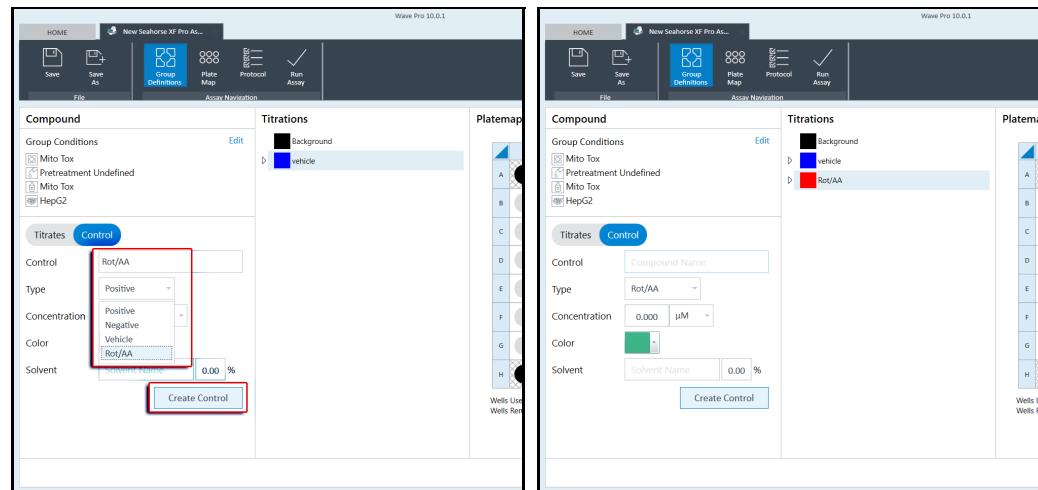
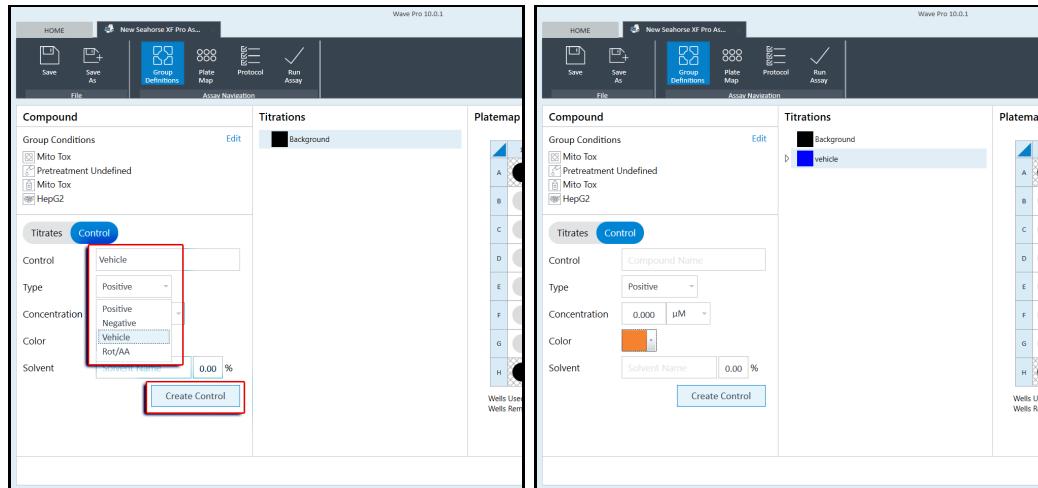
- 2 For the short-term XF Mito Tox assay workflow, the default **Pretreatment** option is used. Click **Continue** to proceed.



4 Assay Template Design and Creation

Define experimental groups and plate layout

- 3 First, create and assign the required control groups: vehicle and Rot/AA.



- 4 Groups can be renamed after creation by double clicking on the group name. To delete a group, double click on the group name and then click the cross on the right.

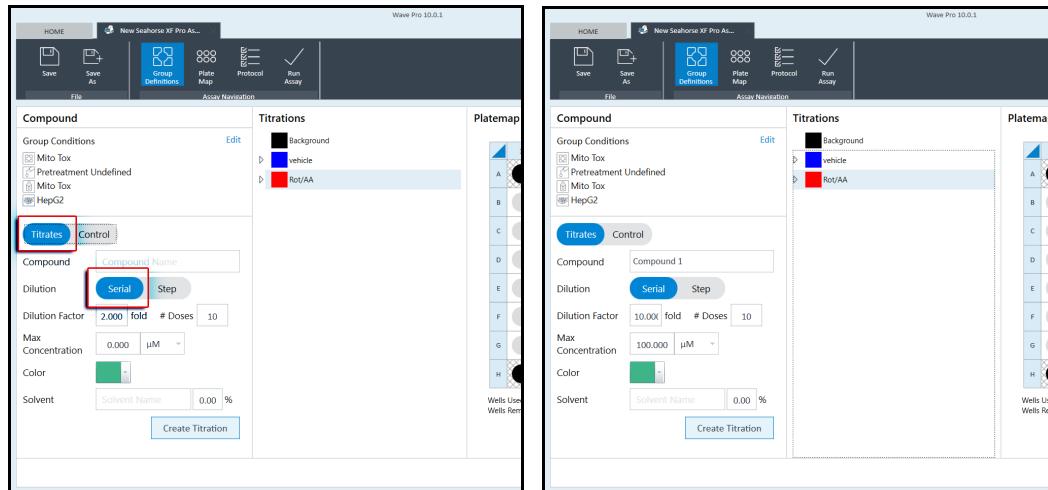
NOTE

The Delete All function at the bottom of the view is used to delete everything created. It will also delete the Group Conditions defined in the previous section.

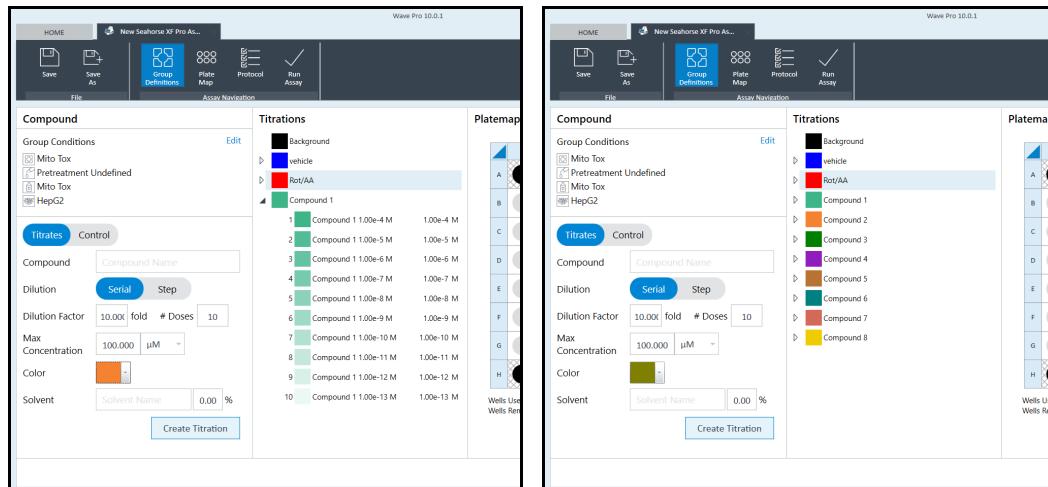
4 Assay Template Design and Creation

Define experimental groups and plate layout

- 5 Once control groups are created, switch to the **Titrates** view. Select either **Serial** or **Step** to start creating a titration series. Enter the compound name, dilution factor, and maximum concentration. Then click **Create Titration**. A serial dilution is generated for the compound. Ensure that the correct concentration and units are assigned at this time, as they cannot be edited later.



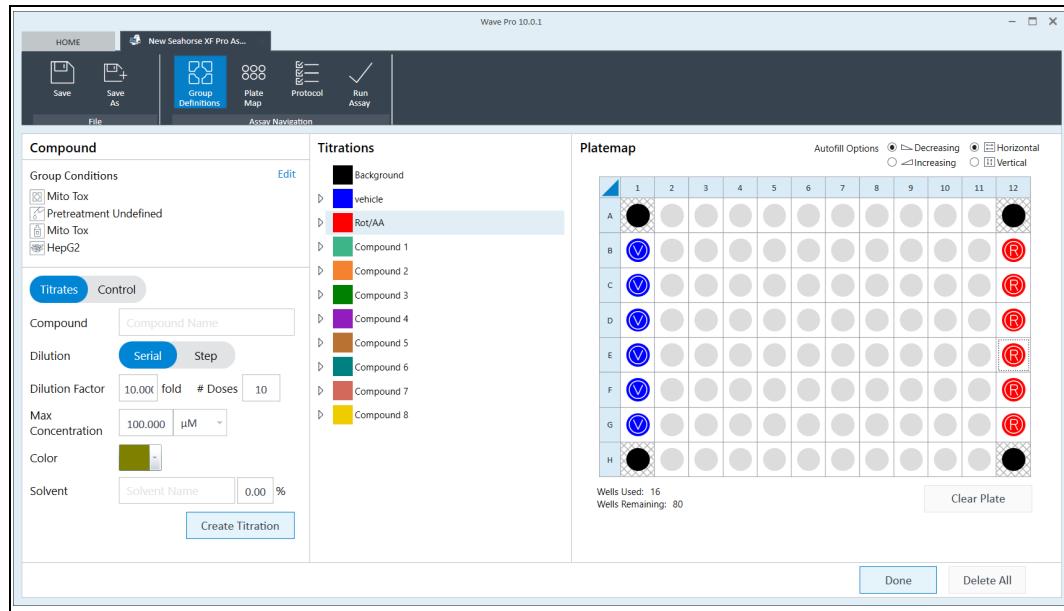
- 6 Use the drop-down arrow on the left of each compound to view all concentrations in a serial dilution. All concentrations are displayed as molar concentrations. Repeat the process for each compound to be tested.



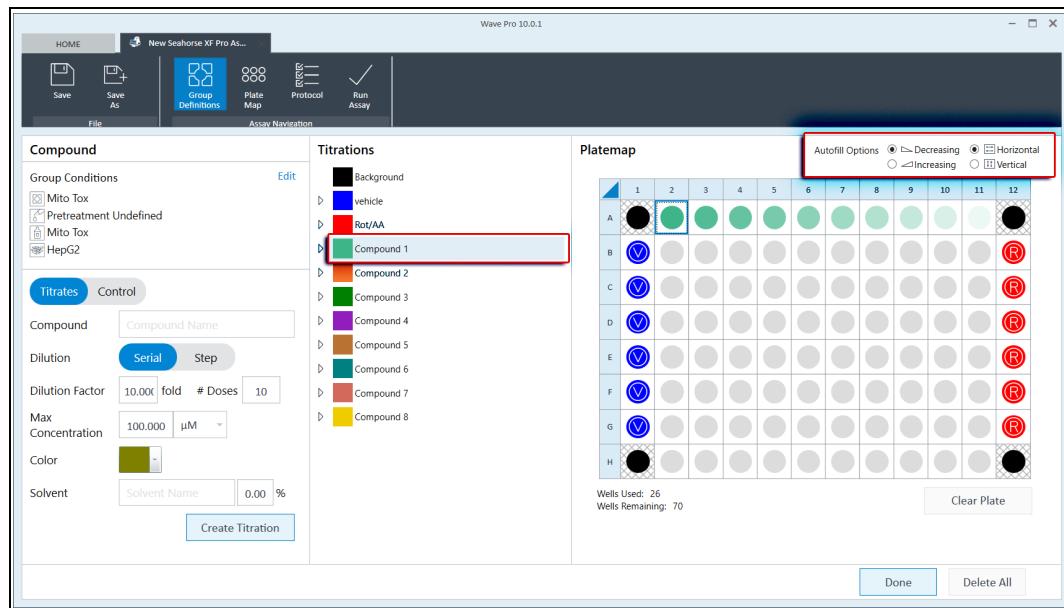
4 Assay Template Design and Creation

Define experimental groups and plate layout

- 7 After all groups are created, first assign control groups to the plate map. Select/highlight the vehicle or Rot/AA group in the group list. Then, move the cursor to the **Platemap**. Click the wells to be assigned. Assigning the vehicle group to column 1 (row B to G) and **Rot/AA** to column 12 (row B to G) is recommended. Ensure that the control types are assigned properly. The MTI parameter cannot be calculated without these control types.



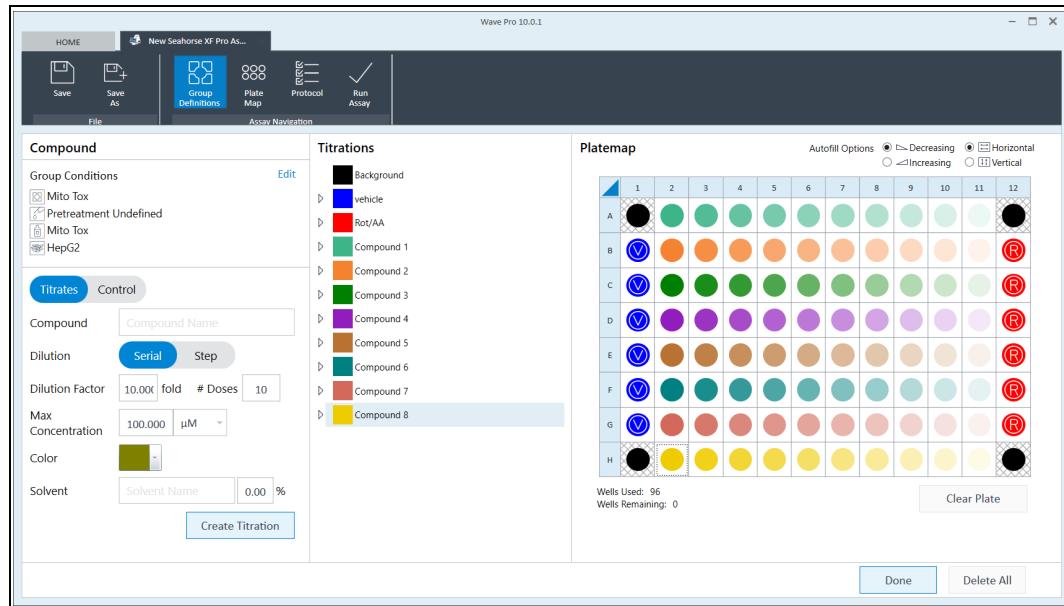
- 8 Compounds can be assigned to the plate map as a complete series using the autofill function. Autofill allows for assignment in horizontal or vertical orientation, and in ascending or descending concentration.



4 Assay Template Design and Creation

Define experimental groups and plate layout

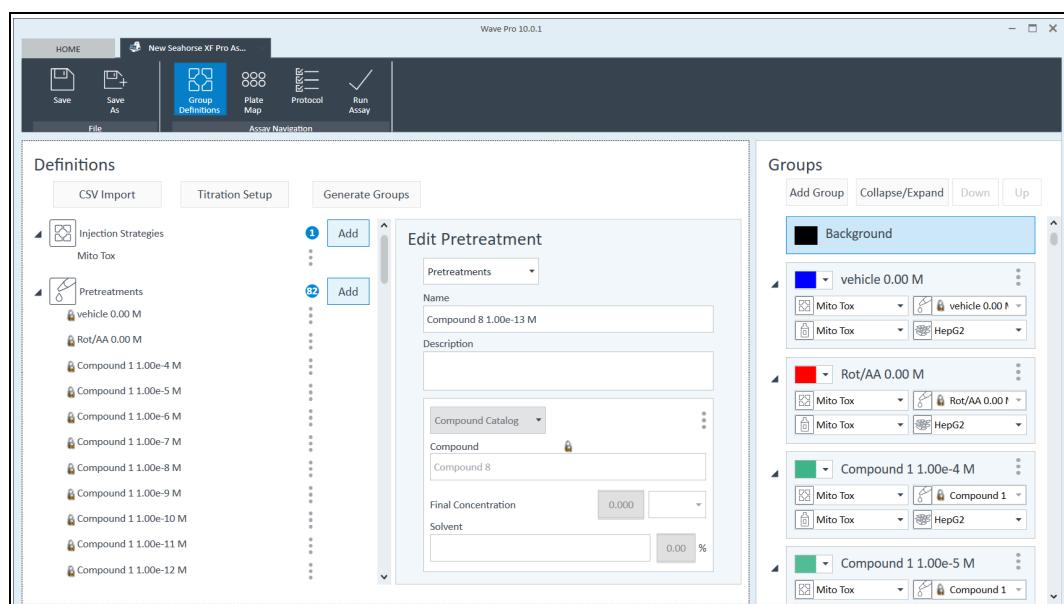
- 9 To assign a dilution series, highlight the parent name in the group list, select the **Autofill Option**, and select the desired location on the plate map. Series will be assigned from left to right or top to bottom on the plate map automatically. The example dilution series here are assigned in a horizontal orientation with a descending concentration. Wells within a titration series have the same color with different shades with the highest concentration being the darkest shade.



- 10 After all dilution series are assigned, click **Done** at the bottom. All controls and titrations are generated as unique groups with a predefined injection strategy, medium type, and cell type.

NOTE

In the Group Definitions view, compound group names (in the right panel) and the pretreatment names cannot be edited.

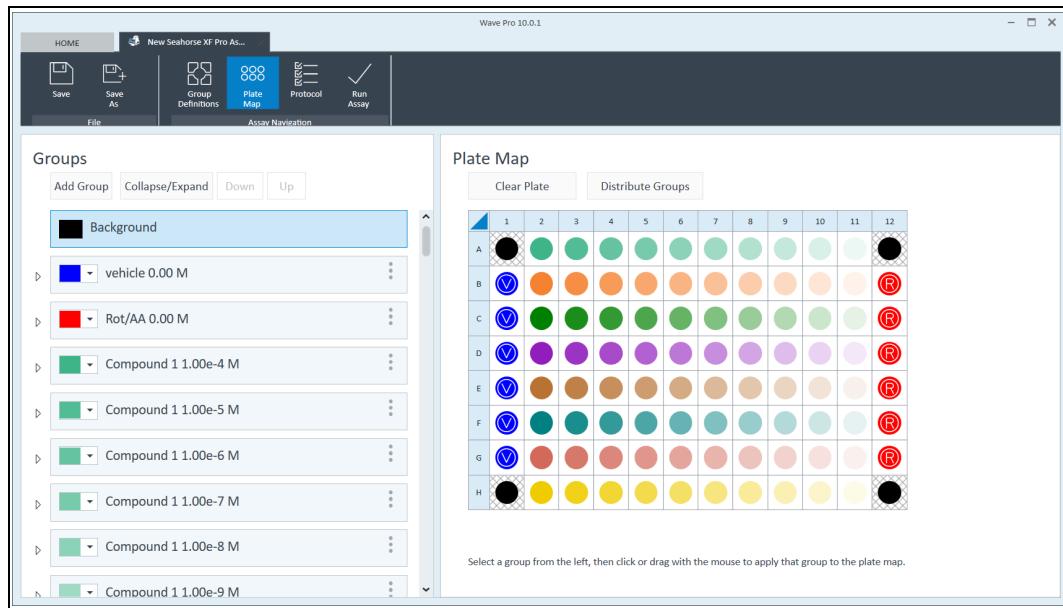


4 Assay Template Design and Creation

Define instrument protocol

NOTE

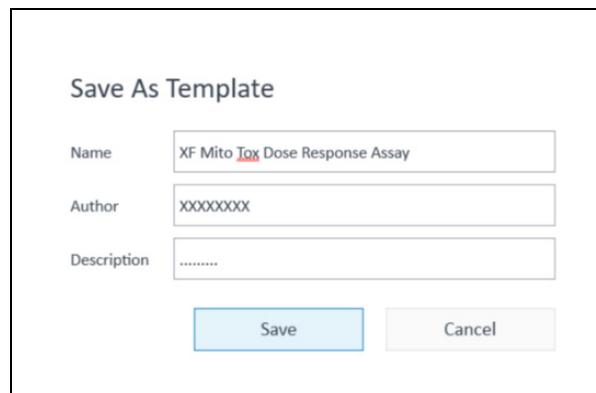
In Plate Map view, the plate map created using the Titration Setup tool can be edited on a well-by-well basis only.



Define instrument protocol

Follow the steps described in the section **“Template Creation for Testing a Small Set of Compounds”** on page 14 to define the instrument protocol [page 21](#).

The template creation is now complete. Click the **Save** symbol at the top left, name the template, and click **Save** in the dialog box.



Upload Assay Template

If an assay template is created using Seahorse Wave Pro software, follow these steps to upload the custom assay template to the XF Pro Controller software.

- 1 Open the XF Pro Controller software on the XF Pro analyzer.
- 2 Insert a USB drive (containing the custom assay template) in the front USB port or connect to a network drive containing the custom assay template.
- 3 Click **Import** (bottom of the New Assay view).
- 4 Locate the custom assay template file on the USB or network drive.
- 5 Click **Open** in the Windows dialog box. The imported assay template will be available for selection from the Templates view on the XF Pro analyzer.
- 6 Repeat for the next template, if applicable.
- 7 The imported assay template (or templates) will now be available for selection in the list of available templates.

Assay Workflow

One Day Before Assay (Day 1) 34

Day of Assay (Day 2) 36

Data Analysis 40

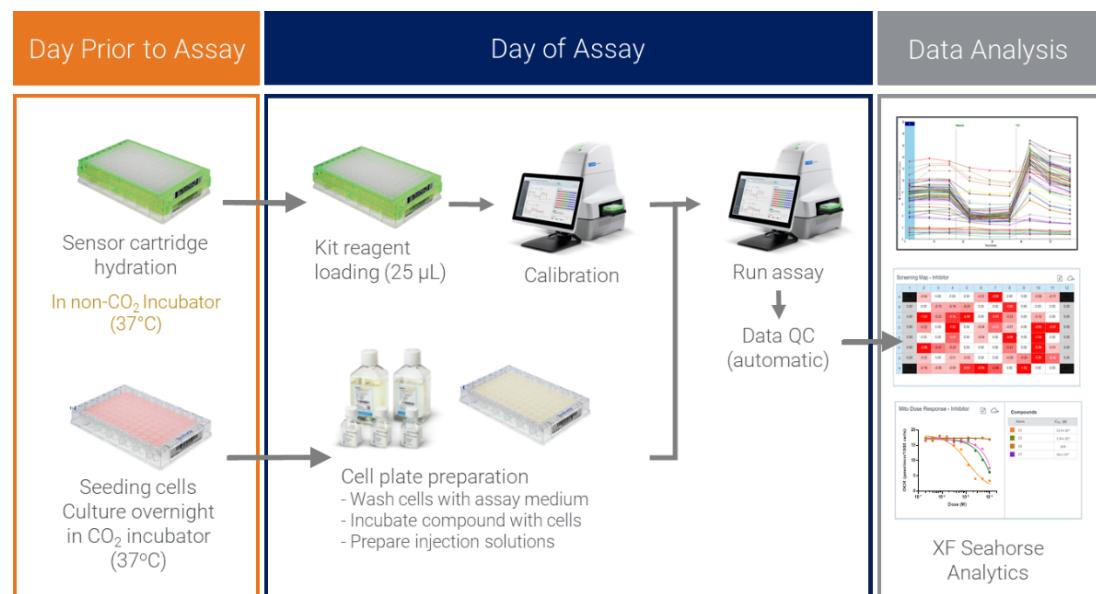


Figure 4. Agilent Seahorse XF Mito Tox assay workflow with the Agilent Seahorse XF Pro analyzer.

5 Assay Workflow

One Day Before Assay (Day 1)

Power up the XF Pro analyzer and ensure it is thermally equilibrated to 37 °C (for a minimum of 2 hours).

Prepare sensor cartridge

- 1 Open an Agilent Seahorse XFe96/XF Pro Extracellular Flux Assay Kit and remove the sensor cartridge.
- 2 Place the sensor cartridge upside down next to the utility plate.
- 3 Fill each well of the utility plate with 200 μ L of calibrant solution and place the XF Hydrobooster on top of the utility plate. Ensure that the hydrobooster has been pressed into place.
- 4 Lower the sensor cartridge onto the utility plate, submerging the sensors in the calibrant or water (see diagram in **Figure 5**)

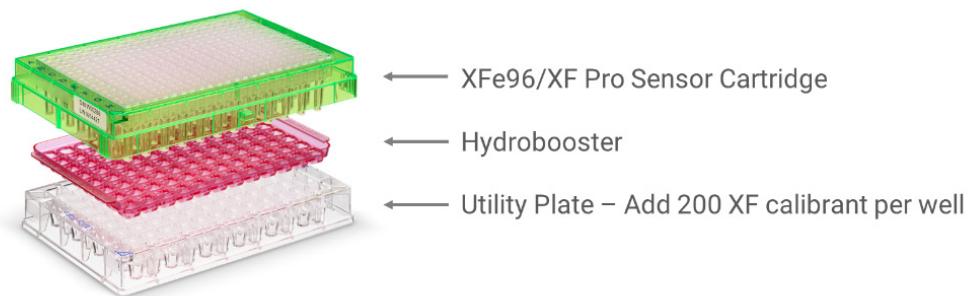


Figure 5. XF sensor cartridge, XF hydrobooster, and utility plate ensemble.

- 5 Verify that the calibrant is high enough to keep the sensors submerged.
- 6 Place the cartridge and utility plate ensemble in a 37 °C, non-CO₂ incubator overnight.

Seeding adherent cells in XF Pro M cell culture plate

- 1 Harvest and resuspend the cells to the desired final concentration and seed in 80 μ L of growth medium. (For example, for 10 \times 10³ cells per well, resuspend cells 10 \times 10³ per 80 μ L = 125 \times 10³ cells per mL).
- 2 Dispense 80 μ L of growth medium into the background wells at the four corners (A1, A12, H1, H12). Dispense 80 μ L of cell suspension into each well. Do not seed cells in background wells.

5 Assay Workflow

Design/create and upload assay template

- 3 Fill the four moat chambers around the wells with water using an eight-channel P1000 pipettor set to 250 μ L (Figure 6). This will result in 1.0 mL volume in each moat chamber (four tips can fit into one moat chamber). If no multichannel pipette is available, fill each chamber of the moat with 1.0 mL water.

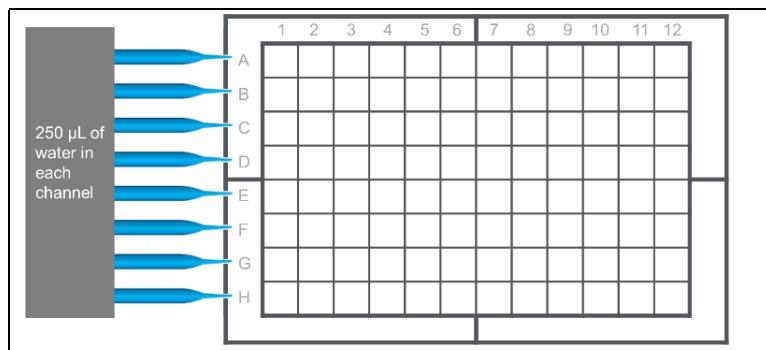


Figure 6. Fill the moats using an eight-channel P1000 pipettor.

- 4 Allow the plate to rest in the tissue culture hood for one hour. This can improve the consistency of the cell monolayer for some cell types. Monitor adherence using a microscope.
- 5 Place the cell plate in a humidity-controlled 37 °C CO₂ incubator and allow cells to grow overnight.

NOTE

For more information about using XF Pro M cell culture plates, refer to Agilent document [5994-4356EN](#). If performing an XF Mito Tox assay with suspension cells, please refer to the seeding suspension cell protocol on the [Agilent Cell Analysis Learning Center website](#) for details.

Design/create and upload assay template

Designing/Preparing assay templates on the day before performing the assay is recommended. Ensure that the templates are uploaded to the XF Pro Controller software on the XF Pro analyzer and are ready to be used. Please follow the instructions provided in [Chapter 4](#) to create assay templates.

Day of Assay (Day 2)

Prepare assay medium

- 1 Prepare the XF assay medium by supplementing 97 mL of Seahorse XF DMEM medium, pH 7.4, with 1.0 mL each of XF glucose, XF pyruvate, and XF glutamine (10 mM, 1 mM, and 2 mM final concentrations in assay media, respectively). These are the recommended initial conditions. The assay medium composition can be modified if desired.

Table 3 Standard XF Mito Tox assay medium.

| Component | Volume (mL) | Final Concentration (mM) |
|---------------------------------------|-------------|--------------------------|
| Seahorse XF DMEM Medium, pH 7.4 | 97 | - |
| Seahorse XF 1.0 M Glucose Solution | 1.0 | 10 |
| Seahorse XF 100 mM Pyruvate Solution | 1.0 | 1 |
| Seahorse XF 200 mM Glutamine Solution | 1.0 | 2 |

- 2 Warm up to 37 °C.

NOTE

No pH adjustment to the assay media is necessary when standard XF supplement concentrations are used.

Prepare test compounds, vehicle, and rot/AA solutions

Important: Use the compounds on the same day that they are reconstituted. Discard any remaining compound solutions. Do not refreeze and re-use.

- 1 Prepare 2x solution for all test compounds solutions using assay medium. For example, if the final concentration to be tested is 100 µM, then prepare a 200 µM solution of test compounds.
- 2 If test compound solutions contain DMSO or other solvents, prepare 5 mL of XF assay medium containing the same final concentration of DMSO or other solvents. This is the vehicle control solution.
- 3 Remove one foil pouch and the decapper from the Seahorse XF Mito Tox Assay Kit box. Remove the Rot/AA vial (red cap) from the pouch.
- 4 Prepare 1.0 µM of Rot/AA solution (2x) by first adding 400 µL of assay medium to the vial. Vortex to dissolve the compounds. Then transfer all 400 µL to a larger vial and add a further 5 mL of assay medium. The total should be 5,400 µL.

NOTE

If the test compound solution contains DMSO, it is recommended that the final DMSO concentration in the wells should be less than 1%. There is no need to include DMSO in Rot/AA solution.

5 Assay Workflow

Prepare cell plate with adherent cells and pre-incubate with test compounds

Prepare cell plate with adherent cells and pre-incubate with test compounds

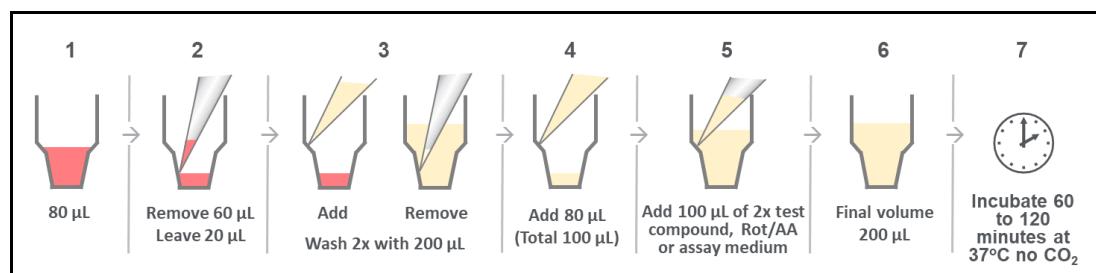


Figure 7. XF Pro M cell plate preparation and test compound pre-incubation. Leave 20 μ L of medium at each wash step to minimize damage to the cell monolayer.

- 1 Remove the cell plate from the cell culture incubator and examine the cells under a microscope to confirm consistent plating, proper cell morphology, and no contamination.
- 2 Wash the cell plate twice with prewarmed assay medium, as indicated in **Figure 7**. After wash, leave 100 μ L medium in each well (see step 4 in **Figure 7**).
- 3 Add 100 μ L of the 2x test compound solution to each well, according to the predesigned template.
- 4 Add 100 μ L of 1.0 μ M Rot/AA solution to the eight cell wells in column 12.
- 5 Add 100 μ L vehicle control solution to the eight cell wells in column 1 and to the four corner background wells.
- 6 Allow the cell plate with the test compounds and Rot/AA to incubate in a 37 °C non-CO₂ incubator for 60 to 120 minutes.

Prepare injection solutions and load to injection ports

- 1 Remove the oligomycin vial (blue cap) and the FCCP vial (yellow cap) from the pouch. Place the tubes in a small tube rack.
- 2 Resuspend the content in each tube with assay medium in the volumes indicated in **Table 4**. Vortex for 30 seconds or gently pipette the solution up and down to dissolve the content.

Table 4 Prepare stock solutions.

| Compound | Cap Color | Assay Medium to Add (μ L) | Stock Solution Concentration (μ M) |
|------------|-----------|--------------------------------|---|
| Oligomycin | Blue | 465 | 135 |
| FCCP | Yellow | 720 | 100 |

5 Assay Workflow

Performing the Mito Tox assay

- 3 Prepare injection solutions using the stock solutions prepared in the above step with the volumes indicated in **Table 5**.

Table 5 Preparation of injection solutions. The concentration and loading volume are determined for a well volume of 200 μ L in a 96-well format before the start of the assay.

| Injection Solution | Stock Solution (μ L) | Assay Medium (μ L) | Port Concentration μ M | Port Concentration Fold | Concentration in Well (μ M) | Loading Port and Volume |
|--------------------|---------------------------|-------------------------|----------------------------|-------------------------|----------------------------------|-------------------------|
| Oligomycin | 300 | 2,700 | 13.5 | 9x | 1.5 | Port A: 25 μ L |
| | 150 | 2,850 | 5 | 10x | 0.5 | |
| | 300 | 2,700 | 10 | 10x | 1.0 | |
| | 450 | 2,550 | 15 | 10x | 1.5 | |
| | 600 | 2,400 | 20 | 10x | 2.0 | |

- 4 Carefully dispense 25 μ L of the oligomycin injection solution to each Port A and 25 μ L of the FCCP injection solution to each Port B.
- 5 Visually inspect the injection ports for even loading. The liquid should be in the port. Make sure there are no residual drops on top of the cartridge.

NOTE

Port A and Port B for wells not intended to be used in the experiment are also required to be filled with 25 μ L of any solution to ensure proper function of the instrument. Correct port loading techniques are important to ensure a successful experiment. Before any experiments, it is recommended that new users read the detailed instructions found under the "Set Up Assay > Loading Solutions" section on the [Agilent Cell Analysis Learning Center](#) website.

Performing the Mito Tox assay

NOTE

If the assay template is not uploaded to the XF Pro analyzer, see the section ["Design/create and upload assay template"](#) on page 35 to upload a template.

- 1 Select the assay template to be used from the list of available templates. Click **Open File** (or double-click the template).
- 2 Review the groups (cell types or conditions), plate layout map, and instrument protocol for the assay. Modify if desired.
- 3 On the review and run display, click **Scan Assay Kit** to display the assay kit information dialog box.
- 4 Use the external handheld barcode wand to scan the barcode on the kit box label (outside). This will automatically add the part number and lot number of the assay kit to this dialog box.
- 5 In the SW ID field, manually type the SW code on the kit box label. Click **Apply**. The software will embed this information in the data file.
- 6 Click **Start Run** when ready, to start the assay.

5 Assay Workflow

Performing the Mito Tox assay

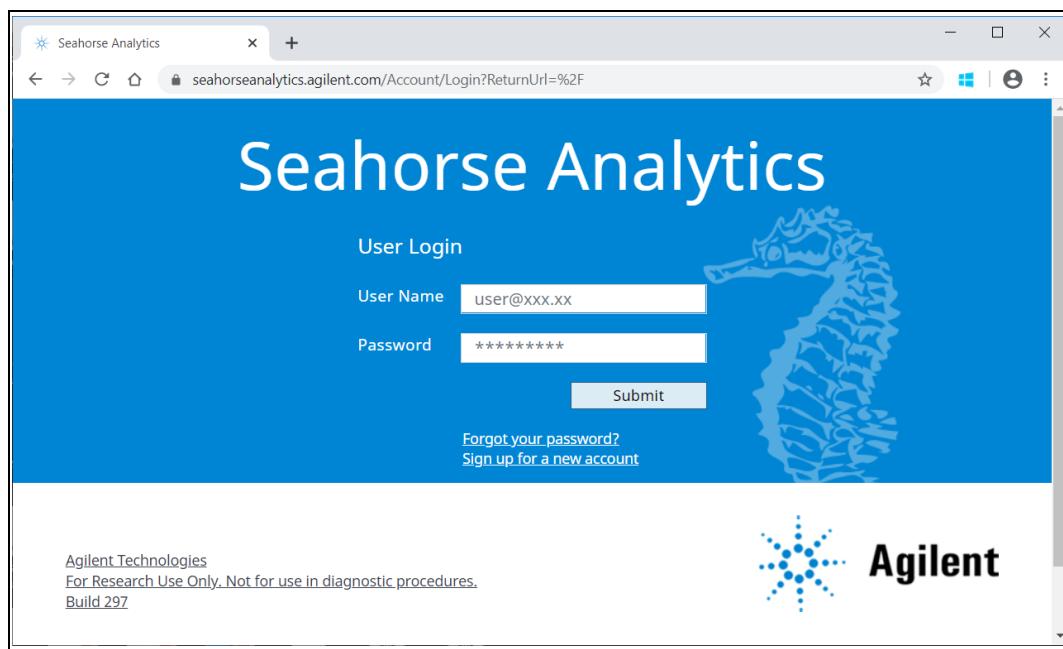
- 7 When prompted, remove the cartridge lid and place the loaded sensor cartridge with the utility plate on the thermal tray of the XF Pro analyzer. Ensure the plate orientation is correct and the cartridge lid has been removed. Then, click **I'm Ready**. Calibration takes approximately 15 to 30 minutes.
- 8 After completing the calibration, the XF Pro Controller displays the **Load Cell Plate** dialog box. Click **Open Tray** to eject the utility plate and load the cell plate. Ensure the lid is removed from the cell plate before loading.
- 9 Click **Load Cell Plate** to run the assay.

Data Analysis

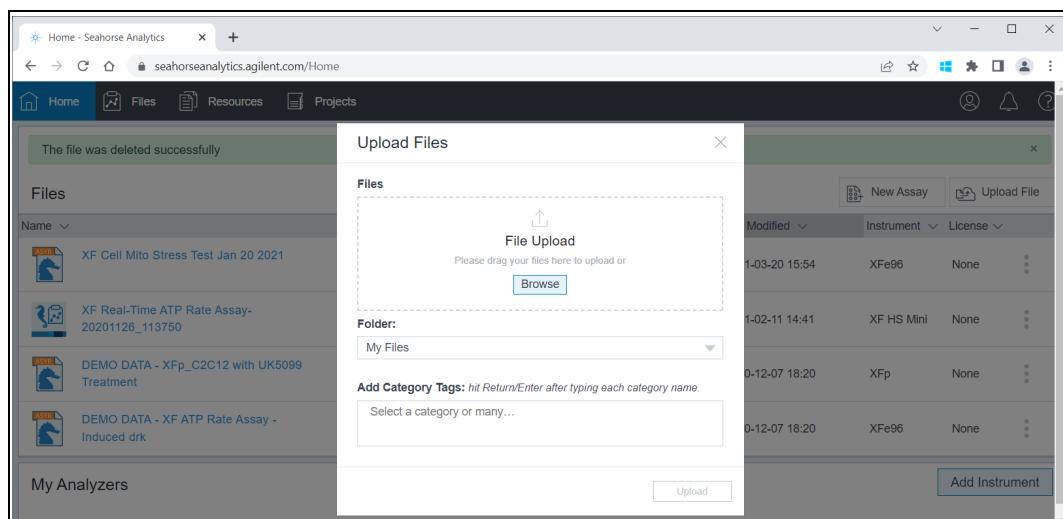
Data analysis and result reporting of the XF Mito Tox assays can be easily performed using Agilent XF Seahorse Analytics, a web-based software platform with dedicated assay companion views. These software features automatically calculate MTI and Z' values, and present the MTI values in heat maps or bar charts. Dose-response curves are plotted and IC₅₀ or EC₅₀ values are calculated. This allows one to streamline the data analysis workflow and quickly obtain organized and shareable summary reports. For details about parameter calculations, please refer to the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵ An Agilent Seahorse XF Discovery software license (for the XF Pro and XF Flex analyzers) and a valid assay kit are required to access these analysis views.

Follow the steps below to use the relevant assay widgets in Seahorse Analytics:

- 1 To register or log in to your Seahorse Analytics account, visit:
<https://seahorseanalytics.agilent.com>.



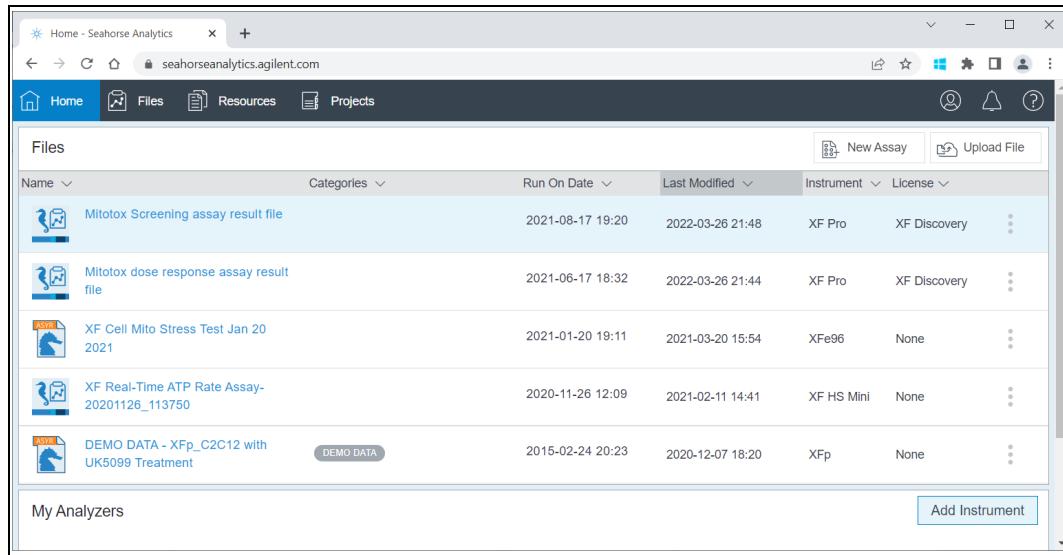
- 2 Upload the assay result file to your account.



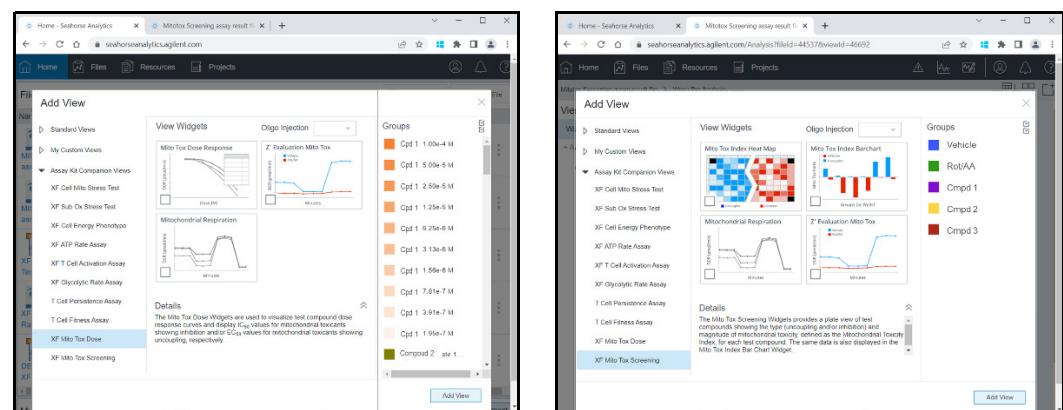
5 Assay Workflow

Data Analysis

- 3 Click the assay result file and the **Add View** dialog box will appear.



- 4 Under the **Assay Kit Companion Views** menu, select the **XF Mito Tox Screening** analysis view if the result file is from a screening experiment, or select the **XF Mito Tox Dose** analysis view if the result file is from a dose-response assay.



- 5 Select the desired analysis widgets in the View Widgets window, ensure the **Oligo Injection** is set to the correct position (default is injection 1), then click Add View at the bottom.
- 6 Review automated Data Quality tests to verify data integrity by clicking the exclamation button at the top right of the window.
- 7 Proceed to data examination.

For additional information about using Seahorse Analytics, visit the [Agilent Cell Analysis Learning Center](#) website. For examples of application and resulting data, please refer to the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵

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6 XF Mito Tox Assay: Alternative Workflows

The schematic diagram in Figure 3 describes three possible workflows for performing the XF Mito Tox assay. All are identical except for when the test compound is provided to the cells. The "pre-incubation" workflow provides test compounds to the cells when the cell culture medium is exchanged for XF assay media, allowing the short-term (~60 to 90 minutes) compound effects to be assessed. This is the recommended (and often simplest) method to use when first performing the XF Mito Tox assay.

As an alternative, the use of injection ports to deliver the compound *in situ* may be used (Figure 3, workflow A). This acute injection method offers the advantage of kinetically tracking any acute effects of the compound in real time. When using this workflow, some adjustments to the procedures are required.

- If an acute injection is desired, adjust the starting volume in each well to 175 μ L in the last wash step (step 6 in Figure 7), as the final volume before the start of the assay.
- For the test compounds to be injected, prepare 8x solutions (to achieve 1x final concentration in the well after the injection), and load 25 μ L of each test compound solution into the respective Port A of the XF sensor cartridge.
- 25 μ L of 8x Rot/AA solution should be loaded into Port A of the Rot/AA control group wells. 25 μ L of oligomycin and FCCP solutions should be loaded to Port B and C, respectively, of all wells.

The XF Mito Tox assay may also be adapted for longer-term compound exposure (hours to days, Figure 3C). While this offers the ability to assess effects of long-term compound treatments, it does require provision of the compound during cell culture. The compound should also be provided (again) in the assay media during exchange with culture media.

- If investigation of longer-term compound effects is required, determine the length of compound exposure, and provide the desired concentration of each test compound during cell culture.
- Rot/AA may be provided to Rot/AA control group wells during cell culture (similar to test compounds), but must be freshly provided to the Rot/AA control group wells upon exchange of culture media for assay media.
- Once the cells have been exposed to the compounds for the desired time, follow instructions for the "pre-incubation" workflow (Figure 3B and described above).

Note that the primary variables to consider in this assay are the concentration of the compound (or compounds) and the length of time the cells will be exposed to the compound (or compounds). Please see Table 2 on page 10 and the associated discussion in the Agilent application note by Kam *et al*⁵ for considerations of each Mito Tox assay workflow shown in Figure 3.

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Frequently Asked Questions

What cell type was used to develop and validate the XF Mito Tox assay workflow?

HepG2 cells, commonly used for assessing drug-induced mitochondrial toxicity, were used to develop and validate the XF Mito Tox assay workflow.

Can the XF Mito Tox assay workflow be used with other types of cells?

Yes, other types of cells may be used. The basis of the XF Mito Tox assay is the XF Cell Mito Stress Test, which has been used successfully with multiple cell types, including cell lines, primary cells, and suspension cells. However, optimization of assay conditions, including cell seeding density and FCCP concentration, is required. These optimization experiments may be performed with the XF Cell Mito Stress Test.

Can I change the composition of the assay media?

Yes. Initial XF Mito Tox assays are recommended to be performed in standard XF assay conditions (see **Table 3** on page 36). Alternative assay medium may be used, including alternative base media, substrates and/or other supplements. However, optimization of assay conditions, including cell seeding density and FCCP concentration, is required.

Can I add BSA and/or serum to the XF Mito Tox assay media?

Yes. BSA and/or serum may be included in the XF Mito Tox assay media and used in the cell culture wells. However, BSA or serum-containing media should not be used in the injection ports. When BSA or serum is included in XF assay media, optimization experiments must be performed, including cell density and FCCP concentration. The presence of BSA or serum can often affect compound potency, and higher concentrations of compound (for example, FCCP) may be required to elicit optimal responses.

Do I need to condition the cells in galactose medium to perform the XF Mito Tox assay?

No, galactose is not required in the cell culture medium nor in the XF assay medium.

How long should the cells be exposed to test compound before running the XF Mito Tox assay?

This is a key decision for the investigator. It is recommended to begin with the short-term workflow, providing compounds to the cells 1 to 2 hours before performing the assay. This is sufficient for studying shorter-term compound effects on mitochondrial function. If investigating long-term compound effects is desired (4 to 72 hours), compound solutions must be provided in the cell culture medium. It is highly recommended that the compounds are also provided at the same concentration after culture medium is exchanged for XF assay, just before performing the assay. Caution must be taken when interpreting results from longer-term exposure of compounds, as they may have complex downstream effects that involve interactions between mitochondrial toxicity and cytotoxicity. For further discussion, please see the Agilent white paper by Rogers *et al.*⁴ and the application note by Kam *et al.*⁵

7 Frequently Asked Questions

Do I need to include both Vehicle Control and Rot/AA Control groups in every assay?

Yes. These control groups are required to calculate MTI values and subsequently, IC₅₀ and EC₅₀ values in dose-response assays. They are also used to calculate the assay performance metric Z' values.

Why are the control groups referred to as Vehicle Control and Rot/AA Control groups and not positive or negative controls?

When calculating an uncoupler MTI and associated Z' values, the minimal Oligo OCR of the vehicle control group is the negative control and the maximal FCCP OCR of the vehicle control group is the positive control. Conversely, when calculating an inhibitor MTI and associated Z' values, the maximal FCCP OCR of the vehicle control group is the negative control, and the minimal FCCP OCR of the Rot/AA control group is the positive control. In other words, the vehicle control group can serve as either positive or negative controls depending on the MTI value being calculated. To avoid confusion, the terms negative or positive controls are not used for these control groups in this context.

Can the inhibitor MTI value go below –1?

Yes, but rarely. Typically, most inhibitors show MTI values between 0 and –1. MTI values of test compounds may go slightly below –1 (which is, by definition, the level of inhibition seen in the Rot/AA control group). In these cases, the compound is likely an extremely potent mitochondrial inhibitor or may be exerting cytotoxic effects.

Can the uncoupler MTI value go above 1?

Yes. Typically, most uncouplers show MTI values between 0 and 1. MTI values of test compounds may go slightly above 1 (which is, by definition, the level of uncoupling in the vehicle group in response to FCCP). This could suggest a potent uncoupler; however, caution should be taken to ensure the concentration of FCCP is optimized and elicits maximal respiration (OCR). This is because using suboptimal FCCP concentrations may result in MTI values significantly greater than 1.

Can a negative MTI value be calculated for uncoupling?

Yes. This can occur when a strong inhibitor decreases the Oligo OCR to a level below the Oligo OCR of the vehicle group (which serves as negative control for uncoupling). In this case, the MTI value is reported as 0.

Can a positive MTI value be calculated for inhibition?

Yes. This can occur when a strong uncoupler increases the FCCP OCR to a level above the FCCP OCR of the vehicle group (which serves as negative control for inhibition). This results in a positive MTI value for inhibition. In this case, the MTI value is reported as 0.

7 Frequently Asked Questions

Can a compound simultaneously show both negative and positive MTI values at a single concentration?

Yes, a compound may elicit both uncoupling (positive MTI) and inhibitory (negative MTI) responses at the same concentration. This is commonly observed for uncouplers, and downstream dose-response analysis is recommended after initial screening assays to further assess and determine the compound's mode of action. For further information on this topic, see the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵

Can the apparent mode of toxicity change at different compound concentrations or depend on the length of time cells are exposed to the compound?

Yes. This is especially relevant for uncouplers. At low concentrations and after short-term incubation (minutes to several hours), uncouplers typically elicit an increase in Oligo OCR. However, the same compound at higher relative concentrations, and/or after an extended incubation period (hours to days), can elicit a decrease in FCCP OCR (meaning it appears as an inhibitor). Modes of toxicity may also change due to nonspecific effects with increasing compound concentration. For further discussion, please see the Agilent white paper by Rogers *et al.*⁴ and the Agilent application note by Kam *et al.*⁵

Can I use/interpret the ECAR/PER/pH signal when performing an XF Mito Tox assay?

The primary purpose of the assay is to quickly and accurately identify and quantify mitochondrial toxicity. While compound impact may be observed from the extracellular acidification rate (ECAR) or proton efflux rate (PER) signals, it is recommended to exercise caution when making any interpretations based on ECAR or PER signal changes. Follow up assays that specifically measure glycolytic PER must be performed (XF ATP rate assay, XF GRA assay) to ensure confident interpretation in those cases. It should be noted that some test compounds may alter the pH of the XF assay media, affecting the ECAR signal. Viewing the pH level data can help to understand if a significant change in the pH of the assay media has occurred upon compound addition.

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