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MitoXpress Xtra Quick Start Guide

Five Step Workflow

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This Quick Start provides all you need to know from plate reader setup and signal optimization, to sample and compound optimizations, as well as routine assay runs and data analysis. For detailed instructions on product use, please see the MitoXpress Xtra user manual.

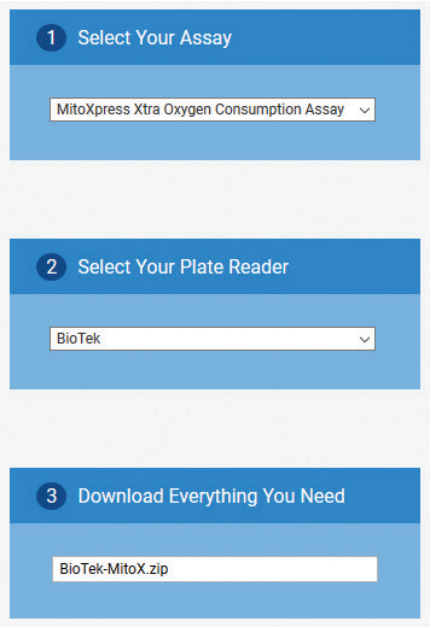
1. Plate Reader Setup

Key steps

1.1 Find your plate reader download packet

1.2 Import protocol/template

1.3 Define the detection mode



Step 1.1

Find your plate reader download packet

Find your Plate Reader online.

The download packet found in the link above contains the required collateral and software templates you will need, including the recommended protocol template files. These files contain default settings for your fluorescence plate reader model, where available, or an instrument setup guide where not available. Separately, it will contain a data analysis template specific to your plate reader control software or, where that is not feasible, provide the data visualization tool (Excel Macro). In addition, the MitoXpress Xtra user guide is provided, containing detailed assay instructions.

Step 1.2

Import protocol/template

Import protocol templates into your plate reader software for easy setup. Open the protocol and define the filter locations specific to your instrument filter wheel/slide/cube and get started.

If a template is not provided, create a protocol in the instrument software, inputting the instrument parameters described in the specific instrument setup guide.

Ensure that software versions are up to date and compatible before starting.

Step 1.3

Define the detection mode

Decide/define which detection mode you intend to employ depending on the plate reader specification used (basic, standard TR-F, and dual-read TR-F). See the user guide contained in the download packet to inform your choice.

Use the recommended detection mode for your chosen plate reader specifications, while ensuring that the correct excitation and emission filters are installed correctly.

In cases where dual-read TR-F detection is recommended but the filters are not available, it is possible to employ the standard TR-F detection as an alternative. This is done using the monochromator rather than filters for excitation and emission wavelength selection.

2. Signal Optimization

Key steps

2.1 Read; 20 minutes at 37 °C

2.2 Calculations

- i. S:B ratio (>3:1)
- ii. Signal level (10 to 20% max. instrument intensity (RFU))
- iii. Lifetime signal level (22 to 26 µs)

2.3 Adjustments

2.4 Confirmation (repeat read)

Step 2.1

Plate reader signal optimization

Measure a cell-free signal optimization plate for verification of the protocol template/instrument settings implemented in Step 1. This can be done with a quick assessment of the signal-to-blank (S:B) and signal level (RFU).

- The recommended S:B is >3:1
- The signal level must be ~10 to 20% maximum intensity (RFU) on the plate reader (~15% of saturation). Otherwise, the signal will overflow/saturate the instrument.

Usually the default protocol parameters will provide a suitable S:B and RFU; however, in the unlikely event it is outside these recommendations, simple adjustments can be made, found in Step 2.3.

Step 2.1.1

Plate preparation

Prepare and read a signal optimization plate using the plate layout in Figure 1. MitoXpress probe, media, and HS mineral oil preparation are described in the MitoXpress Xtra user guide.

	1	2	3	4	5	6	7	8	9	10	11	12
A	MX probe	MX probe	MX probe	MX probe								
B	Blank	Blank	Blank	Blank								
C												
D												
E												
F												
G												
H												

MX probe: 10 µL MitoXpress probe + 90 µL Media + 100 µL HS mineral oil
Blank: 100 µL Media + 100 µL HS mineral oil

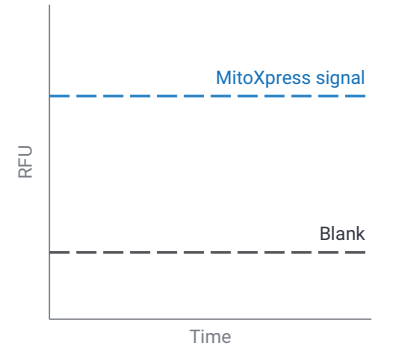
Figure 1. Signal optimization plate layout.

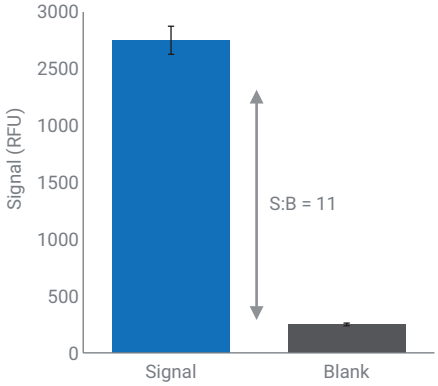
Note: It is important that the media/sample, HS mineral oil, microplate, and plate reader are prepared and maintained at the desired assay temperature, 37 °C. The HS mineral oil should be warmed to 37 °C before the assay for easier use.

Tip: For consistent, reliable, and quick HS mineral oil dispensing, a **repeater type pipette** is recommended (using a 1.25 mL syringe tip or 2 mL combitip). Prepare the repeater syringe tip by trimming ~3 to 4 mm off the tip at a 45° angle. Remove the internal nozzle from the oil dropper bottle and slowly pick up the prewarmed HS mineral oil (avoid pipetting up and down, as this can cause bubbles) and dispense 100 µL into each well at an angle of ~45°, allowing the oil to flow down the side of each well.

Pipetting tips are found on page 36 of the MitoXpress Xtra user guide.

Key steps





Step 2.2

Calculations

Perform calculations i. to iii. using one of the following data analysis options:

(a) Plate reader software analysis/templates

(b) Agilent Data Visualization Tool

Review the raw results and calculate the following:

i. **Signal:blank ratio:** Using average RFU values measured at the 20-minute time point, calculate signal RFU (row A)/blank RFU (row B). Ensure that the S:B ratio is >3. If using a TRF detection mode: S:B >5 to 10 is achievable.

ii. **RFU signal level:** Using RFU signal at the 20-minute time point, the measured RFU signal level for the MX probe samples should be in the range of ~10 to 20% of the maximum instrument intensity (RFU). See the plate reader user manual or software help section to identify the arbitrary maximum/saturated RFU signal level for the given plate reader model.

iii. **Lifetime signal (µs) level:** MitoXpress assay signal (21% O_2) ~22 to 26 µs.

Note: This calculation is only applicable when using the advanced dual-read TRF detection mode on a filter-based plate reader.

Decision point:

- If the recommended S:B ratio, signal level, and lifetime values (where applicable) are successfully achieved, we recommended proceeding to Step 3.
- If, however, the recommended S:B ratio, signal level, or lifetime values are not achieved, we suggest proceeding to Step 2.3 Adjustments.

Key steps

2.1 Read; 20 minutes at 37 °C

2.2 Calculations

- i. S:B ratio (>3:1)
- ii. Signal level (10 to 20% max. instrument intensity (RFU))
- iii. Lifetime signal level (22 to 26 µs)

2.3 Adjustments

2.4 Confirmation (repeat read)

Step 2.3

Adjustments for improved signal detection (S:B and signal level)

If necessary, based on the calculation in Step 2.2, the following adjustments are recommended:

i. **Parameter review and confirmation**
First, review and confirm that all instrument parameters were inputted correctly and not changed or omitted. See the instrument setup guide, MitoXpress Xtra user guide, and original template protocol.

ii. **Focal (Z-) height optimization**
To improve RFU signal level and S:B for a given microplate format and volume, check and adjust the focal height setting manually or automatically depending on reader capability. See the plate reader user manual. Only applicable where focal height adjustment is available.

iii. **Gain or photomultiplier tube (PMT) setting adjustments**
The protocol templates employ fixed gain or PMT settings throughout the kinetic measurement. *Auto Gain, Dynamic, or Variable Gain should not be used*.

- If signal level is too high (>20% max. intensity), decreasing the gain or PMT setting is recommended.
- If signal level is too low (<10% max. intensity), increasing the gain or PMT setting is recommended.

Note: If using advanced dual-read TRF detection mode, attention should be on the RFU signal measured from Window 1 (W1), (30 µs delay read/window), when targeting 10 to 20% max. intensity. Also, both W1 and W2 TRF settings must use identical gain settings; this is critically important to a successful lifetime signal-based assay.

iv. **Flash number, or number of pulses**
Increase the flash number (no.) value to yield improved signal stability. Highest flash no. will yield longer cycle time.

Note: Default TRF (time-resolved fluorescence) settings (delay time and integration/window times) should not be adjusted.

Additional adjustments: Details on these can be found in the Troubleshooting section of the MitoXpress Xtra user guide. These include switching to bottom read detection, removal of phenol red and serum from media, or increasing MitoXpress probe concentration by 5 µL/well, or a combination of all options.

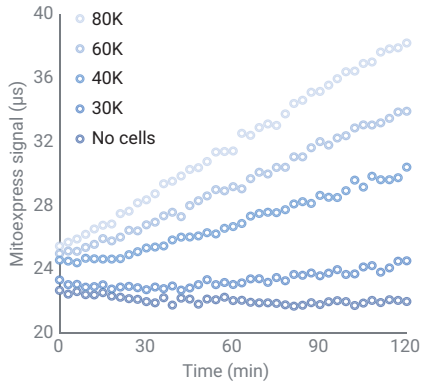
Step 2.4

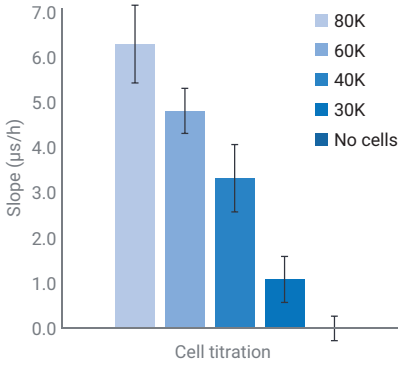
Confirmation

Repeat Step 2.1 and 2.2 using the same signal optimization plate to verify the success of any adjustments made in Step 2.3. Once confirmed, move to Step 3.

3. Sample Optimization

Key steps





Determine the relative oxygen consumption rate (OCR) from a range of cell densities. This is essential for identifying the suitable seeding density required for detectable metabolism (relative OCR) for a given cell type/model under fixed conditions. *The cell density required is cell type-specific; these concentrations are general guidance only.*

Step 3.1

Adherent cell titration plate

3.1.1 Day before measurement

Plate cells with the following seeding densities: 30,000, 40,000, 60,000, and 80,000/200 µL media overnight (n = 4) using a suitable TC⁺ 96-well microplate. The suggested plate layout is provided in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	80K	80K	80K	80K								
B	60K	60K	60K	60K								
C	40K	40K	40K	40K								
D	30K	30K	30K	30K								
E	0K	0K	0K	0K								
F	Signal control	Signal control	Signal control	Signal control								
G	**Blank**	**Blank**	**Blank**	**Blank**								
H												

Cell plating densities (X *1000 cells/well): 60K is 60,000 cells/well
Signal control: Media + MitoXpress probe + HS mineral oil
Blank: Media + HS mineral oil

Figure 2. Adherent cell plate layout.

*** Optional:** Positive signal control (GOx) can be added to row H (n = 4). For example, GOx plus MitoXpress in media, plus HS mineral oil (positive signal control). *Add 10 µL of 1.5 mg/mL Gox stock/well. (GOx well concentration: 0.15 mg/mL).

Tip: Allow the plate to rest on the bench for 30 minutes at room temperature after the cells have been pipetted onto the plate. This will help ensure an evenly dispersed monolayer to help minimize plate edge effects.

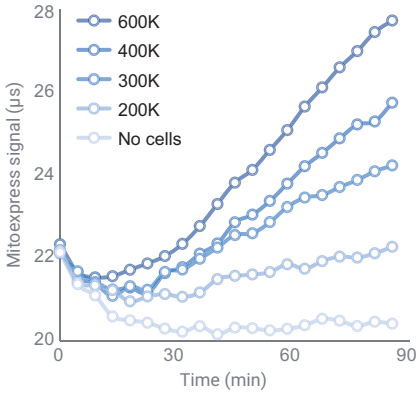
3.1.2 Day of measurement

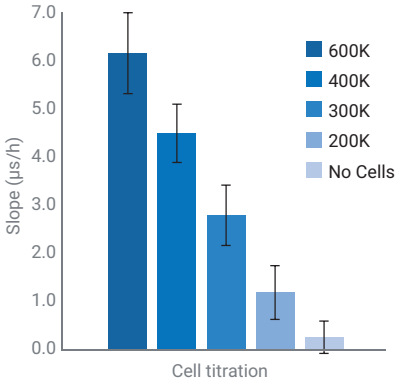
Prepare the cell assay plate as per user manual guidelines, including the appropriate control and blank samples.

- See the previous recommendation (page 4) to use a repeater type pipette for optimum HS mineral oil addition.
- Measure the cell titration plate kinetically at 37 °C for two hours using minimum cycle or interval time (one minute), immediately after the HS mineral oil layer is applied.

Note: Blank and signal control samples are always required (minimum n = 2) and should be included in all routine MitoXpress Xtra assays.

Key steps





Step 3.2

Suspension cell titration plate

3.2.1 Day of measurement

Plate cells with the following density; 200,000, 300,000, 400,000, and 600,000/90 µL media (n = 4) using a suitable 96-well microplate. See Figure 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	600K	600K	600K	600K								
B	400K	400K	400K	400K								
C	300K	300K	300K	300K								
D	200K	200K	200K	200K								
E	0K	0K	0K	0K								
F	Signal control	Signal control	Signal control	Signal control								
G	Blank	Blank	Blank	Blank								
H												

Cell plating densities (X *1000 cells/well): 400K is 400,000 cells/100 µL
Signal control: Media + MitoXpress probe + HS mineral oil
Blank: Media + HS mineral oil

Figure 3. Suspension cell plate layout.

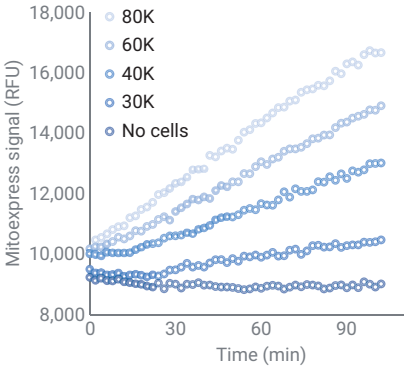
***Optional:** Positive signal control (GOx) can be added to row C (n = 4). For example, GOx plus MitoXpress in media, plus HS mineral oil. Add 10 µL of 1.5 mg/mL GOx stock/well. (GOx well concentration: 0.15 mg/mL).

Tip: Temperature control and temperature equilibration of the microplate and cell suspension during plate preparation is important in minimizing inconsistent temperature across the plate. Use a plate heat block set to 37 °C where possible.

3.2.2

Prepare the cell assay plate as per the MitoXpress Xtra user guide instructions, by adding 10 µL MitoXpress/well, including the appropriate control and blank samples.

Key steps



Step 3.3

Calculations

Perform these calculations to identify a suitable cell density for use in routine assays. A suitable cell density is one that yields either of the criteria in i and ii.

Cell density criteria:

i. A signal fold increase (RFU increase) of ~1.3 to 2-fold over a 60-minute period

ii. A MitoXpress Xtra lifetime signal slope of >4 µs/h if using advanced dual-read TRF detection mode

If a higher minimum level of signal change (O_2 consumption) was preferred, an even greater cell density could be chosen.

Apply data analysis using one of the options provided:

(a) Plate reader software analysis/templates

(b) Agilent Data Visualization Tool

For more detail, see Step 5 for data analysis recommendations.

3.3.1

To accurately measure slope (signal increase), choose a suitable time range over which the RFU signal is analyzed. When using the data visualization tool, visualize the kinetic signal profiles in the chart and *choose an appropriate start time and end time for slope calculation that best captures the linear portion of the signal profiles from all samples*. Typically, this would be after any initial RFU signal decrease (temperature equilibration of the sample) during the initial 10 to 20 minutes.

3.3.2

Review the results and determine the measured rates (slope of signal increase) of all the titrated samples. Calculate average and standard deviation, and plot average slope values versus cell density, including the probe-only signal control.

3.3.3

Determine the cell density that achieves the **signal fold increase** or **lifetime signal slope** criteria above.

Decision point:

- If the recommended cell density criteria are successfully achieved, we recommended proceeding to Step 4.
- If the criteria are not achieved, we recommend performing the adjustments described in Step 3.4

Step 3.4

Adjustments for improved O_2 consumption (OCR)

This is essential for identifying the suitable cell density required for detectable metabolism (relative OCR) in each cell type/model under fixed conditions. The cell density required is cell type-specific.

If the cell density criteria are not achieved, we recommend performing some or a combination of the following adjustments:

i. Re-evaluate the results, ensuring that the correct time range is being used for analysis (avoid temperature equilibration) and that there are no outliers, incorrect calculations, or data corrections being applied incorrectly.

ii. Repeat the titration experiment, ensuring the following:

- Better temperature control at 37 °C of the media, assay test plate, plate reader microplate chamber, and HS mineral oil during plate preparation.
- Accurate control of media and sample volume.

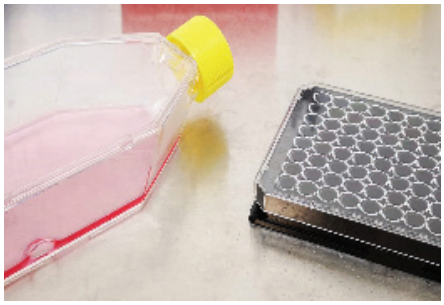
iii. Repeat the titration experiment with an **increased cell density** (amount of sample), repeating Step 3 again.

iv. Repeat the titration experiment with a **decreased well volume, 75 or 50 µL**. (for example: 50 µL/well, 10 µL MitoXpress plus 40 µL media). **Do not decrease well volume below 50 µL/well (96-well microplate). The volume of HS mineral oil added does not change**.

After any adjustments/repeat titration experiments, perform Step 3 calculations again to ensure that cell density criteria are achieved. Only once this is done should you proceed to Step 4.

4. Running the Assay

Key steps



Step 4.1

Example control compound treatment (and FCCP optimization)

Prepare a control compound plate as follows:

i. Seed the plate with the optimum cell density (X) identified in Step 3.

ii. Prepare and add control compound treatments described in Step 4.1.2 and in Figure 4.

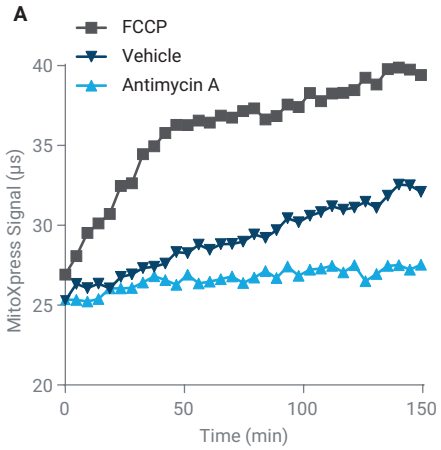
	1	2	3	4	5	6	7	8	9	10	11	12
A	FCCP 2.5 µM	FCCP 2.5 µM	FCCP 2.5 µM	FCCP 2.5 µM								
B	FCCP 1.25 µM	FCCP 1.25 µM	FCCP 1.25 µM	FCCP 1.25 µM								
C	FCCP 0.625 µM	FCCP 0.625 µM	FCCP 0.625 µM	FCCP 0.625 µM								
D	FCCP 0.313 µM	FCCP 0.313 µM	FCCP 0.313 µM	FCCP 0.313 µM								
E	1 µM Rot/Antimycin	1 µM Rot/Antimycin	1 µM Rot/Antimycin	1 µM Rot/Antimycin								
F	DMSO control	DMSO control	DMSO control	DMSO control								
G	Signal control	Signal control	Signal control	Signal control								
H	Blank	Blank	Blank	Blank								

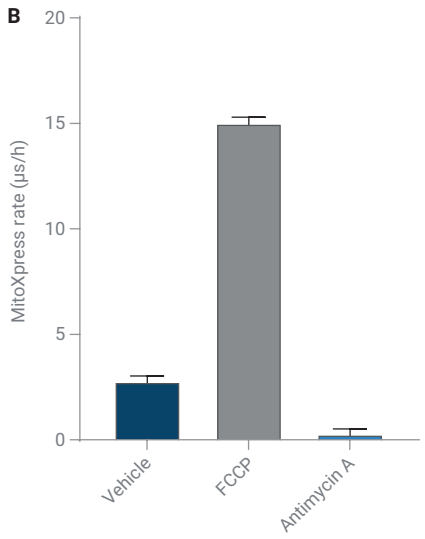
Cell Plating Density: Rows A to F, columns 1 to 4 (for example, adherent cell type: 50,000/well)
FCCP: A range of concentrations from 0.3 µM to 2.5 µM
Rot/Antimycin: 1 µM final concentration
Signal Control: Media + MitoXpress probe + HS mineral oil
Blank: Media + HS mineral oil

Figure 4. Cell compound treatment plate layout. For suspension cell type, plate the cells on the day of measurement, for example 400,000/100 µL.

***Optional:** Positive signal control (GOx) can be added to G3 and G4 wells (n = 2). For example, GOx plus MitoXpress in media, plus HS mineral oil. Add 10 µL of 1.5 mg/mL GOx stock/well. (GOx well concentration: 0.15 mg/mL).

Key steps





Step 4.1.1

Day before measurement cell plate preparation

Plate cells with the optimum cell density:

Adherent cells

Plate cells at X *1000 cells/200 µL media overnight (n = 36), using a suitable TC⁺ 96-well microplate. *For example, 50,000 cells/200 µL for HepG2 cells row A to F, column 1 to 4 of microplate*. See Figure 4.

Tip: Allow the plate to rest on the bench for 30 minutes at room temperature, as before.

Suspensions cells

Prepare on the day of measurement as described in Figure 4, rows A through F, columns 1 through 4 of the microplate. *For example, 400,000 cells/100 µL for HL60 cells rows A to F, columns 1 to 4 of microplate*.

Tip: Prepare a master mix stock of suspension cells in media plus the MitoXpress Xtra probe, for best consistency.

Step 4.1.2

Control compound preparation

Prepare 100X stock concentrations in vehicle (DMSO):

- FCCP (positive control). A serial 1:2 dilution dose response is advisable between 2.5 and 0.3 µM, final concentration.
- Rotenone/Antimycin is the recommended negative control. 1 µM final concentration.

Note: The information in 4.1.1 and 4.1.2 is general guidance only. Response may be cell-type dependent. Adjust compound concentrations as required.

Tip: For initial investigative experiments with compounds, measuring at 37 °C with minimum interval time (one minute) is recommended for a minimum of two hours.

Step 4.1.3

Assay plate preparation

Prepare the assay plate per the user manual instructions, including the appropriate signal control and blank samples. Adding 1 µL of the control compounds (100X) stock to the appropriate wells (see Figure 4). Then add 100 µL of HS mineral oil (warmed to 37 °C) to all test wells, before starting assay measurement.

Tip: Prepare a master mix of MitoXpress Xtra probe plus media for the consistent and simplified addition of MitoXpress reagent. Prepare suitable volume and excess to dispense 100 µL/well.

For consistent, reliable, and quick HS mineral oil dispensing, see pipetting tips found in the user manual on page 36.

5. Data Analysis

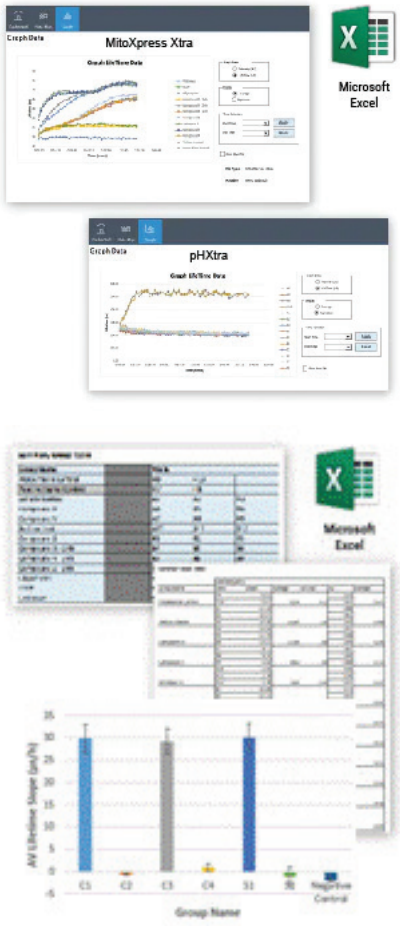
Key steps

5.1 Apply data analysis, and select a suitable time range for slope analysis

5.2 Data analysis tool options available:

a) Plate reader software analytics templates

b) Agilent Data Visualization Tool



Step 5.1

Guideline for applying data analysis tools to raw data

Using either (a) plate reader software analysis/templates or (b) the Agilent Data Visualization Tool, all of which are contained in the download packet retrieved in Step 1, or here:

a) Find your Plate Reader

b) Agilent Data Visualization Tool

To accurately measure slope (signal increase), choose a suitable time range over which the RFU signal is analyzed. When using the data visualization tool, visualize the kinetic signal profile chart and **choose an appropriate start and end time for slope calculation that best captures the linear portion of the signal profiles from all samples**. Typically, this should be after any initial RFU signal decrease (temperature equilibration of the sample) during the initial 10 to 20 minutes.
For plate reader software templates, there are specific functions/buttons for correctly choosing start and end times for the slope calculations. Look for these in the slope or the V max reduction/calculation step in each case, and consult the vendor analysis software user manual if necessary.

Step 5.2

Data analysis tool options

Plate reader software analytics templates
Plate reader software analysis templates or data analysis as part of software protocol files are available from the following plate reader vendors:

1. BioTek - Gen5 protocols (.prt)
2. BMG Labtech - MARS data analysis templates (.MTF)
3. Molecular Devices - SoftMaxPro protocols (.spr)

Agilent MitoXpress and pH Xtra data visualization tool
Carefully read to the Data Visualization Tool (DVT) user manual before use. Save and export the results in the designated compatible format, (csv, .txt file) from the plate reader software.

The data visualization tool is a Microsoft Excel Macro that automatically transforms experimentally derived fluorescence data into kinetic signal curves, applies time range slope calculation, with slope and endpoint results conveniently tabulated and illustrated as charts.

Simple approach

- Export results into the correct DVT-compatible file format: .TXT or .CSV.
- Load the .TXT or .CSV file into DVT.
- Follow the directions for annotation, data visualization, and time range selection (slope calculation) in the Graph tab.
- Summary data table and charts output available.

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