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Optimization of the Agilent MitoXpress Xtra Oxygen Consumption Assay Using BioTek Gen 5 Software

Step-by-Step Instructions

Gen5™

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Step-by-step instructions to maximize the functionality of the MitoXpress Xtra template (AgilentXO2) for BioTek Gen5. This guide includes Plate Reader Setup and Signal Optimization, Sample and Compound Optimization, as well as Running the Assay and Data Analysis. For detailed instructions on product use, please see the MitoXpress Xtra user manual.

Figure 1. Top level workflow for Gen5 and MitoXpress Xtra.

1. Instrument Setup

Key steps

1.1 Cube installation
1.2 Template download
1.3 Signal optimization
1.4 S:B assay
1.5 Data reduction

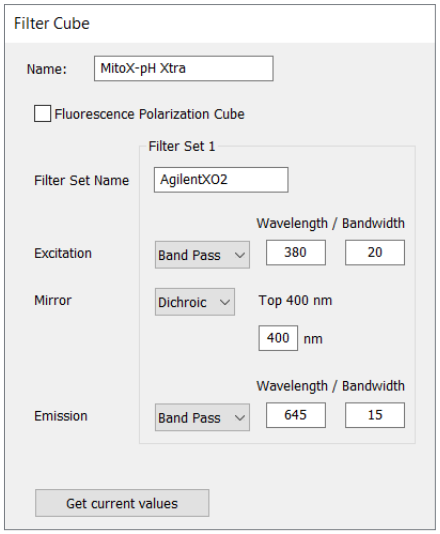

Figure 2. Instructions on how to enter a new filter cube into the Optics Library.

1.1 Cube installation

To run this assay on a Synergy H1, a filter cube is required. The BioTek filter cube for MitoXpress Xtra (p/n 8040587) or the combination cube for MitoXpress Xtra and pH-Xtra (p/n 8040594) can be used.

- https://www.biotek.com/products/accessories/fltr-blk-mitoxpress-xtra-asby/
- https://www.biotek.com/products/accessories/combination-filter-cube-for-mitoxpress-and-ph-xtra-glycolysis-assays/

Check that the cube values match the settings in Figure 2, then define the cube in Gen5 and on the reader.

Open the hinged door on the front of the instrument and place the filter on the filter rail. If the filter cube is oriented correctly (the nonlabeled side facing the chamber), gently slide the cube into the chamber. You will feel a magnet engage the cube.

To add the filter cube into the **Optics library, select System > Optics Library > Filter Cubes**. Then select Add and enter a name for the filter cube. This name will become available for selection in the **Procedure** section for **Protocols** and **Experiments**.

1.2 Template download

Download the file “AgilentXO2.prt” from:

https://www.biotek.com/resources/sample-files/agilentxo2/

The template can be loaded in the task manager in **Experiment > Create using an existing protocol or Protocols**.

Tip: Save protocols as independent files when a modification has been made for ease-of-use and for reference in future experiments. Modifications to the procedure will result in current data being replaced to preserve the audit trail.

Key steps

1.1 Cube installation

1.2 Template download

1.3 Signal optimization

1.4 S:B assay

1.5 Data reduction

Materials required

- Agilent MitoXpress Xtra Oxygen Consumption Assay (part number MX-200-4)
- Standard microplates
- DMEM Medium (part number 41965039)

1.3 Signal optimization

Signal optimization is the first step to a successful experiment. Fluorescence-based assays have a background signal in blank wells that comes from various sources, such as media, plastic, and the detection itself. The signal-to-blank (S:B) ratio is used to ensure that the probe signal is stable and detectable above the background. A robust S:B is required for good quality data.

We recommend that assays have an S:B of at least 3. Probe concentration and the correct gain settings on the S:B will only need to be checked once before carrying out experiments. These settings should be checked and adjusted if the assay medium, plate type, or assay volume change.

Wells that contain MitoXpress Xtra without any cells are referred to as **Signal Control (SC)**. Signal from these wells should be stable after any initial temperature equilibration and can measure around 20% of the maximum scale value, ~20,0000 RFU (Synergy H1 scale: 100 to 99,999 RFU).

These instructions briefly describe how to test S:B using the AgilentXO2 template. See the MitoXpress Xtra Quick Start Guide[1] or the MitoXpress Xtra User Guide[2] for complete instructions.

i. In the Gen5 task manager, select **New Task > Experiments > Create using existing protocol**, then select **AgilentXO2**.

ii. Save the protocol as *Signal Optimization*.

iii. Modify the plate layout under the protocol tab. Place **Blanks (BLK)** and **Signal Controls (SC)** appropriately (Figure 3).

iv. Check that the settings in **Procedure** match Figure 4.

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

Figure 3. Plate map for S:B check.

Key steps

1.1 Cube installation

1.2 Template download

1.3 Signal optimization

1.4 S:B assay

1.5 Data reduction

1.4 S:B assay

i. Solubilize MitoXpress Xtra in 1 mL of media.

ii. Add the following reagents to the microplate:

   a. BLK: 100 µL medium

   b. SC: 90 µL media + 10 µL MitoXpress Xtra

iii. Carefully add 100 µL of HS oil to each well.

iv. Start the assay, **Read New** and allow to run for 20 to 30 minutes.

v. (Optional) select **SC** wells for autogain.

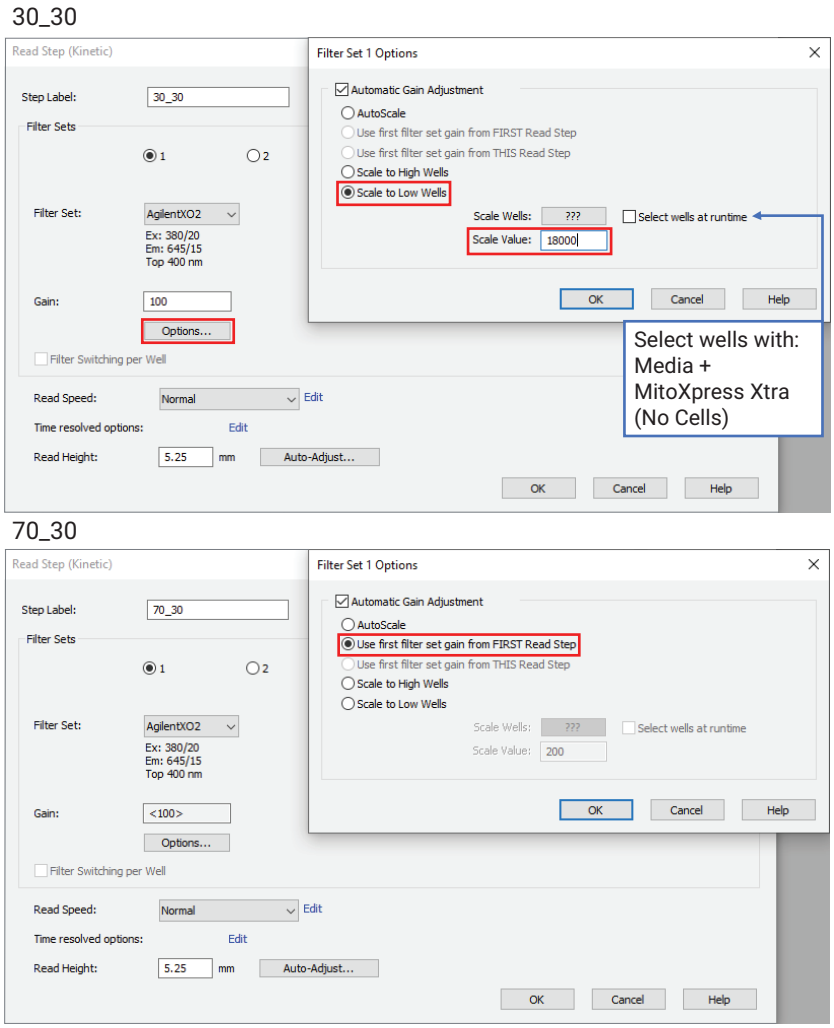

Figure 4. Setting up automatic gain adjustment.

Key steps

1.1 Cube installation

1.2 Template download

1.3 Signal optimization

1.4 S:B assay

1.5 Data reduction

1.5 Data reduction

After the assay, Gen5 will calculate S:B and display values in wells A1 and B1. The two S:B values correspond to each of the dual-reads required in Lifetime mode.

Check that the formula, DS1.SC/DS1.BLK and DS2.SC/DS2.BLK, are entered correctly for A1 and B1, respectively, in the **Data Reduction** section.

Select **Data: S:B** from the drop-down menu. The S:B should be calculated from the ~20 minute time point. The signal should be stable following any initial temperature settling period.

If the S:B is less than 3, check the gain settings and plate map. If the signal is still lower than 3 increase the probe concentration in the well and see the MitoXpress Xtra Quick Start Guide[1] or the MitoXpress Xtra User Guide[2] for detailed troubleshooting steps.

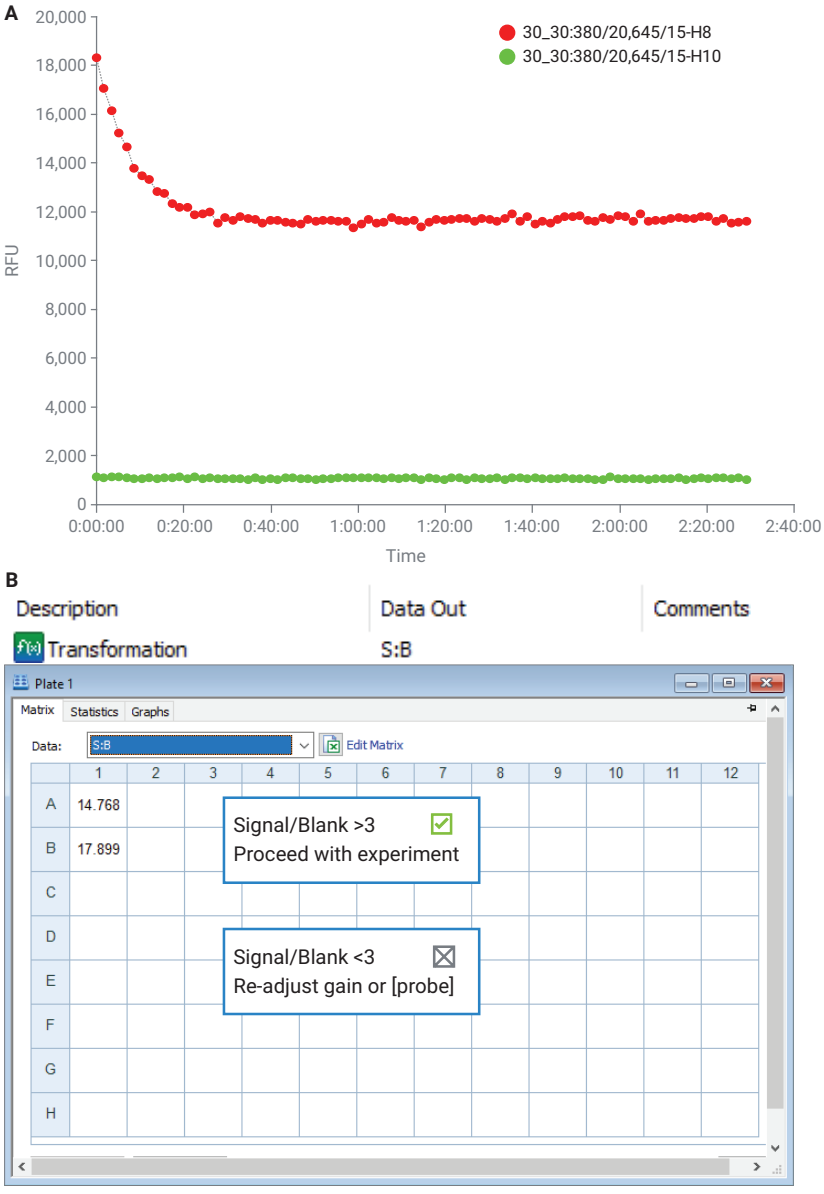

Figure 5. Signal-to-blank ratio over the course of an experiment. Signal-to-blank ratio calculation displayed in plate matrix.

2. Cell number optimization

Key steps

2.1 Cell titration
2.2 Template setup
2.3 Cell titration assay
2.4 Data reduction

Different cell types will respire at different rates. To get the best quality data, we recommend that the optimum seeding density should return a rate >4 µs/h (µs: unit of lifetime). Rates are calculated using the slope of the linear portion of the kinetic graph (see the MitoXpress Xtra Quick Start Guide[1]). Rates of around 4 µs/h generally have a large enough assay window to investigate inhibitors of mitochondria, as well compounds/conditions that can increase oxygen consumption rates.

For most adherent cells, the recommended seeding densities are between 20,000 and 80,000 cells per well. **This step is essential** because performing an initial cell number optimization will save time later and free up resources for key experiments.

Here we demonstrate a titration using HepG2 cells in complete DMEM.

2.1 Cell titration

i. Plate cells across a range of densities (for example, 10,000 to 80,000 cells/well) in complete media and allow them to attach at room temperature before placing the plate in the incubator.

ii. Culture cells overnight.

See Quick Start Guide[1] for suspension cells optimization.

2.2 Template setup

i. In the Gen5 task manager, select **New Task > Experiments > Create using existing protocol**, then select **AgilentXO2**.

ii. Save the protocol as *Cell Optimization*.

iii. Enter a plate map, select **Plate Layout**, then add a standard curve. Select **Add > Standard Curve**, then enter the values for the cell numbers (Figure 6).

iv. Select the wells and drag to populate.

Key steps

2.1 Cell titration

2.2 Template setup

2.3 Cell titration assay

2.4 Data reduction

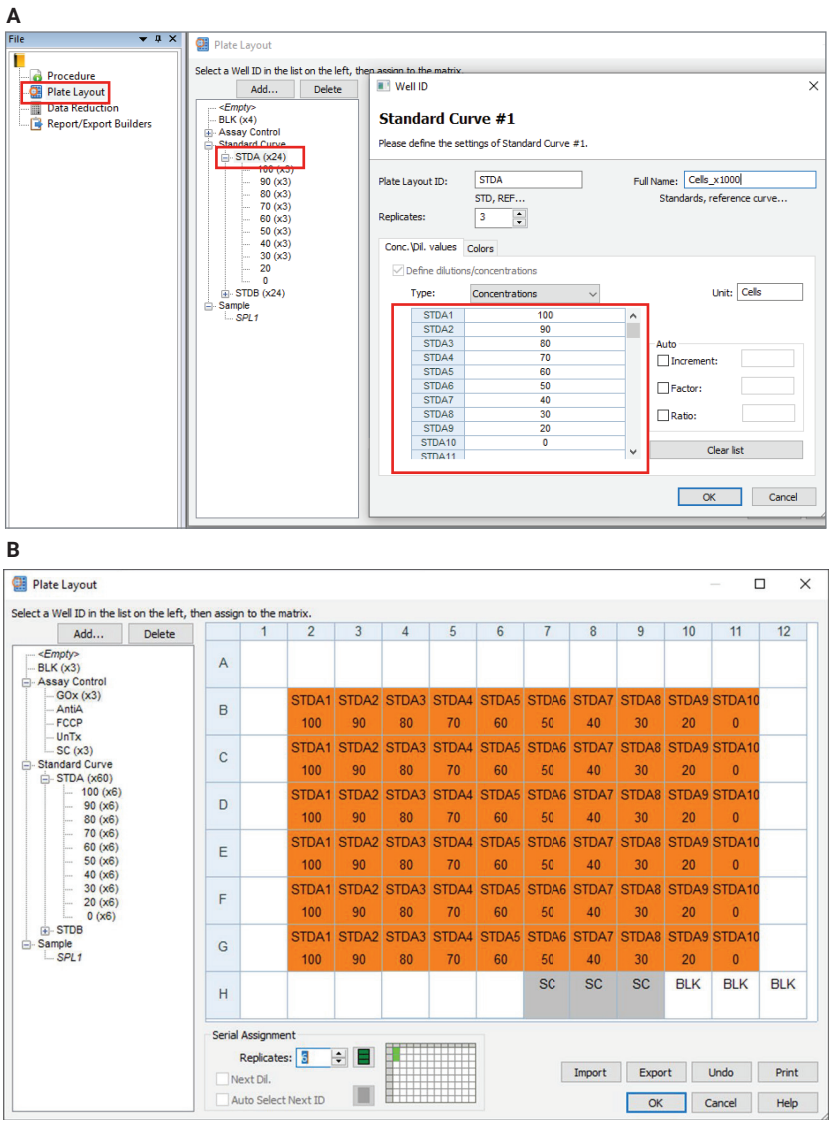

Figure 6. Adding a standard curve for cell optimization and suggested plate layout for optimization assay.

Key steps

2.1 Cell titration

2.2 Template setup

2.3 Cell titration assay

2.4 Data reduction

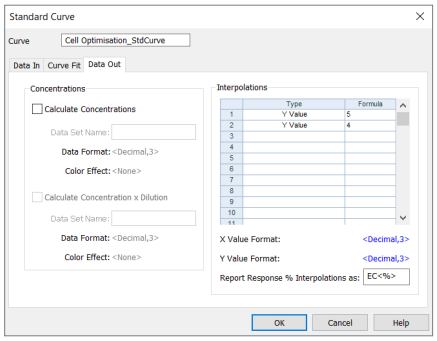

Figure 7. Calculating optimal seeding density from the standard curve.

2.3 Cell titration assay

i. Carefully aspirate the medium and replace with fresh MitoXpress Xtra medium (1x MitoXpress Xtra dissolved in 11 mL media).

ii. Add the following reagents to a microplate:

   a. BLK: 100 µL medium

   b. Samples + SC: 100 µL of MitoXpress Xtra medium

iii. Carefully add 100 µL of HS oil to each well.

iv. Start the assay, select the **Read Now** icon.

v. (Optional) select SC wells for autogain.

2.4 Data reduction

i. To generate a standard curve (Figure 8), select **Data Reduction > Curve analysis > Standard Curve**.

   a. Well ID – STDC

   b. X-Axis Data- Concentrations/dilutions

   c. Y-Axis Data – OCR Max V [Lifetime]

   d. Name the Curve *Cell Optimization_StdCurve*.

ii. To visualize the titration, select the **Graphs** plate view window and display **Cell Optimization_StdCurve** plot (Figure 8).

Using this graph, calculate which cell number generates a rate >4 µs/h. This cell seeding density should be used for subsequent experiments. Alternatively data can also be plotted manually by plotting rate values from the **OCR Max V** table in **Plate View**.

Key steps

2.1 Cell titration

2.2 Template setup

2.3 Cell titration assay

2.4 Data reduction

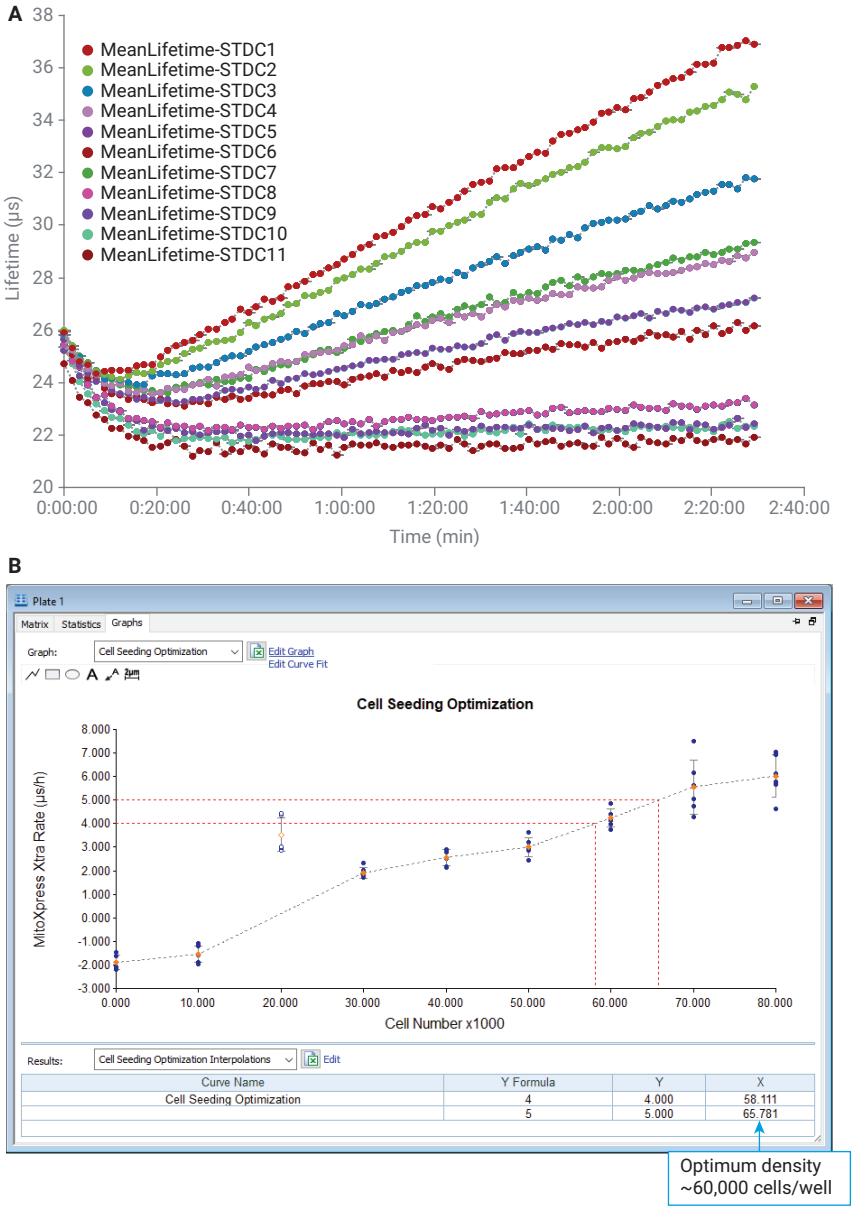

Figure 8. Signal curves (A) for cell optimization assay and calculated rates (B).

3. Mitochondrial function assay

Key steps

3.1 Plate preparation
3.2 Template setup
3.3 Assay preparation
3.4 Mitochondrial assay
3.5 Data reduction and results

Additional materials

- Agilent Seahorse XF Cell Mito Stress Test kit (part number 103015-100)
- Agilent Seahorse XF Glycose/Pyruvate Stress Test (part number 103673-100)
- HepG2 cells (ATCC part number HB-8065)

The MitoXpress Xtra Oxygen Consumption Assay is an optimized solution for assessing mitochondrial function using a BioTek plate reader. When combined with the Agilent Cell Mito Stress Test Kit, several parameters that reflect mitochondrial function can be investigated, including basal, maximum respiration (FCCP), oligomycin treated rates, and non-mitochondrial oxygen consumption (rotenone/antimycin A). The assay provides insight into the mechanism of mitochondrial dysfunction and allows investigation of functional differences among cell types and drug candidates, as well as genetic or biochemical interventions.

Here, we provide a step-by-step guide for testing mitochondrial function from assay setup to data generation. In this example, the pyruvate carrier inhibitor, UK5099, is used to demonstrate the effects of compound treatment on mitochondrial respiration in HepG2 cells.

3.1 Plate preparation

i. Plate cells at the density determined in the previous section (for example, 60,000 cells/well) in growth medium, and allow cells to attach at room temperature before placing the plate in the incubator.

ii. Culture cells overnight.

3.2 Template setup

i. In the Gen5 task manager, select **New Task > Experiments > Create using existing protocol**, then select **AgilentXO2**.

ii. Save the protocol as *Mitochondrial Function Assay*.

iii. Set a plate map, select **Plate Layout,** and place blank and signal control in correct locations.

iv. Add new groups, select **Assay Control**, then name each sample and place them appropriately (see Figure 9). Alternatively enter each condition as a sample, for example, SPL1-4 then label samples using **Plate > Sample ID**.

Key steps

3.1 Plate preparation

3.2 Template setup

3.3 Assay preparation

3.4 Mitochondrial assay

3.5 Data reduction and results

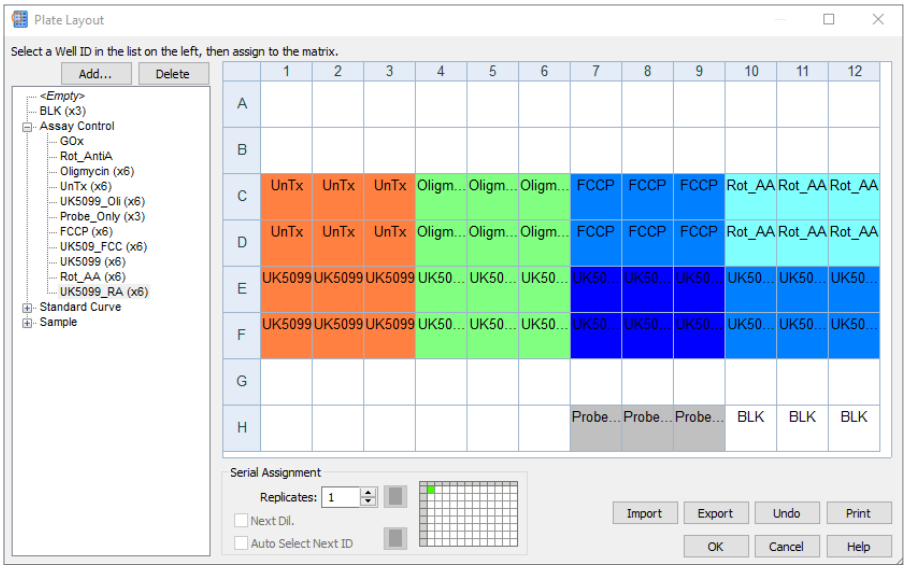

Figure 9. Plate layout for compound testing.

3.3 Assay preparation

i. Prepare the 100x compound stocks (see Table 1).

ii. Place the microplate on a heat block set to 37 °C.

iii. Carefully aspirate the medium and replace with fresh MitoXpress Xtra medium (1x MitoXpress Xtra dissolved in 11 mL medium).

iv. Add the following to each group:

   a. **Blank wells:** 100 µL medium

   b. **Signal control (SC):** 100 µL MitoXpress Xtra medium

   c. **Compound groups:** 90 µL MitoXpress Xtra medium plus 10 µL 10x test compound stocks/vehicle control

Table 1. Recommended concentrations for test compounds.

Compound	100X Stock Volume of Media (µL)	Dilution for 10X Stocks Stock:Media (µL)	10x Stock Concentration (µM)	Final Concentration (µM)
Oligomycin	420	200:1800	15	1.5
FCCP	360	200:1800	20	2.0
Rotenone/Antimycin A	540	200:1800	5	0.5
UK5099	280	200:1800	20	2.0

3.4 Mitochondrial function assay

i. Add 100 µL of prewarmed HS oil carefully to each well and check for air bubbles.

ii. Place the plate into the Synergy H1.

iii. Select **Procedure** to check that gain settings are correct from optimization (or perform autogain).

iv. Select **Read Now**.

Key steps

3.1 Plate preparation

3.2 Template setup

3.3 Assay preparation

3.4 Mitochondrial assay

3.5 Data reduction and results

3.5 Data reduction and results

The Gen5 data reduction tool generates signal change curves and calculates the slope of the linear portion of the curve. Data can then be easily exported to a graph software for display and analysis.

In the **Plate View Matrix**, select **OCR: Max V [Lifetime]**. These values are the rates of MitoXpress Xtra signal change for each well. By selecting wells in **Curves [Lifetime]**, signal trace curves can be displayed in a separate window, as shown in Figure 10. The trendline for the rate calculation should also be displayed in this view.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	8.358	9.055	8.458	2.165	3.343	2.165	21.142	10.072	14.396	2.219	-2.651	3.150
B	10.093	9.735	9.101	2.542	2.945	2.091	34.395	24.454	20.409	2.172	-2.045	3.179
C	8.827	9.087	8.890	2.608	2.947	2.646	36.659	28.371	23.871	-2.213	-2.187	2.581
D	7.318	6.351	7.077	9.886	2.311	3.295	38.487	32.272	27.278	2.283	2.171	2.201
E	8.836	8.156	7.969	3.163	2.637	2.081	37.863	34.077	32.178	-2.983	2.261	2.835
F	9.328	8.855	8.595	2.856	2.405	2.432	38.085	38.450	37.378	*13.552*	2.146	-1.827
G	9.004	7.899	6.481	3.798	4.695	2.371	39.703	36.752	38.162	2.470	2.556	-2.939
H	2.240	-2.845	-2.320	-9.004	8.766	9.187	2.099	1.290	-1.622	?????	?????	?????

B

Well	Well ID	Name	OCR: Max V [Lifetime]	OCR: R-Squared [Lifetime]	OCR: t at Max V [Lifetime]	OCR: Lagtime [Lifetime]
E2	SPL5		8.156	0.896	0:25:08	0:08:42
E4	SPL12		3.163	0.863	0:54:36	0:43:14
C9	FCCP3	FCCP...	23.871	0.964	0:09:32	0:02:14
E12	SPL26		2.835	0.496	0:13:00	0:08:09

Figure 10. Mitochondrial function assay (A) rate data displayed in plate matrix and (B) lifetime signal curves with slope calculations for row E generated in Gen5.

Key steps

3.1 Plate preparation

3.2 Template setup

3.3 Assay preparation

3.4 Mitochondrial assay

3.5 Data reduction and results

Using the data reduction tools provided in this guide rate, data can be pasted directly into graphing software for further analysis (Figure 11).

Untreated or basal rates should be >4 µs/h, depending on the cell type and media.

FCCP treatment uncouples the mitochondria, allowing for maximum respiration in the media condition. Depending on the cell type and conditions, rates are usually around two to four times higher than the basal rates. Optimization of the FCCP concentration is important, as too little will cause incomplete uncoupling, but too much will cause damage to plasma membranes (see the Quick Start Guide[1] for FCCP optimization steps).

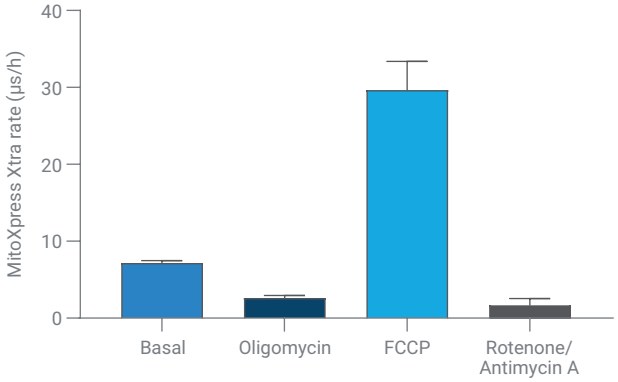

Figure 11. Mitochondrial function assay using MitoXpress Xtra and Agilent Mito Stress test. Rates of change are reflective of oxygen consumption rates in HepG2 cells when treated with mitochondrial modulators.

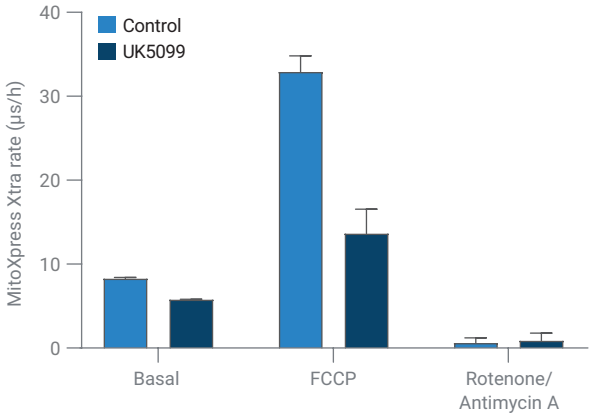

Figure 12. Compound treatment assay using the Seahorse XF Glucose/Pyruvate Oxidation Stress Test Kit shows that treatment with UK5099 deceases basal and maximum respiration rates. The compound restricts access to substrates required for respiration.

4. References

1. MitoXpress Xtra Quick Start Guide: https://www.agilent.com/cs/library/usermanuals/public/user-guide-mitoxpress-xtra-quick-start-guide-cell-analysis-5994-1822en-agilent.pdf
2. Agilent MitoXpress Xtra Oxygen Consumption Assay (HS Method) User Guide: https://www.agilent.com/cs/library/usermanuals/public/MitoXpress_Xtra_Oxygen_Consumption_Assay.pdf

5. Appendix

Tricks and tips

For easier analysis, open and pin multiple plate views to compare the layout with rate data. Use the multiple window button in the top-right corner of the Plate Matrix to do this. This enables users to quickly reference plate maps or any combination of data outputs.

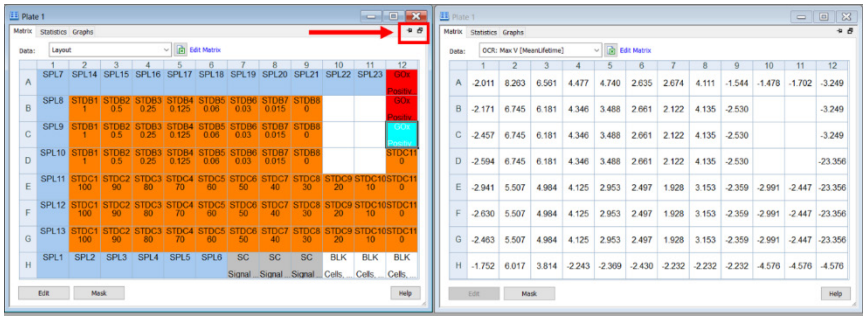

Figure 13. Gen5 allows multiple views from the same data set. Display rates with layout or curve data.

Max slope adjustments

When using Max V for rate calculation, make sure that Gen5 is placing the calculated line as close to the trace line as possible. If the reduced tread line is off, decrease the number of points for the calculation. Check the R^2 value for confirmation (the closer to 1, the better the fit).

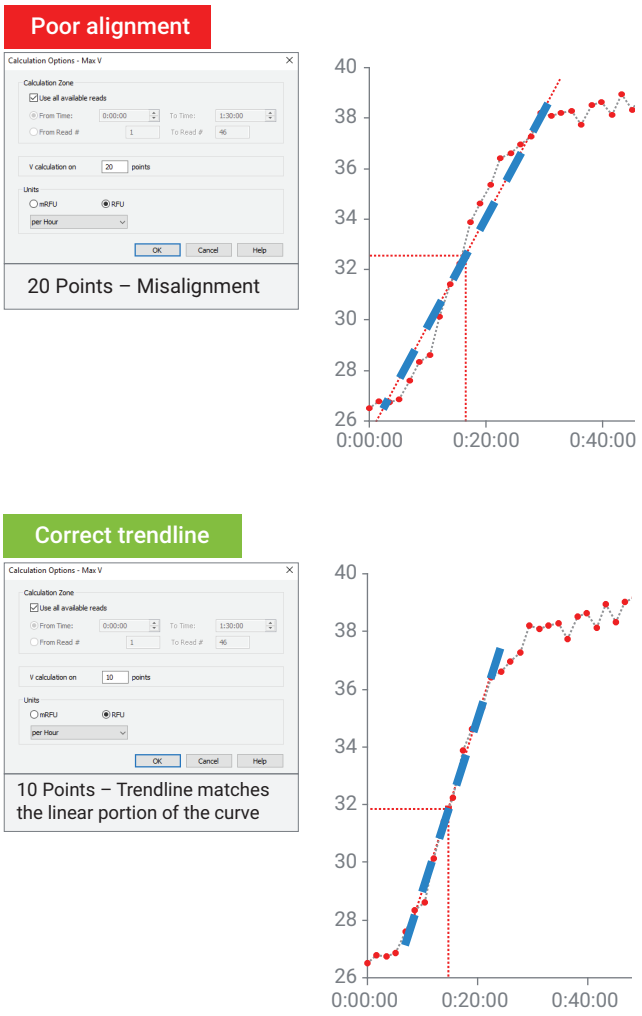

Figure 14. Amount of data points the Max V calculated over can influence the rate. Adjust points to ensure Max V follows the linear portion of the curve.

If Gen5 returns negative values, ensure that the initial temperature equilibration is not being used to calculate "Max V". Negative values should be treated as 0 because this is an artifact of the calculation method (cells are not producing oxygen, noise in the data is being over estimated). If there is a significant decrease in the rates from the Signal Control wells, they may be subtracted from all wells using OCR_SC transformation with the formula: X-SC, applied to all wells, using OCR as data input. This is not always recommended, as it can introduce artifacts from the signal control.

Data reduction quick reference

Data Out	Description	Data In	Formula	Well
S:B	Transformation	DS1	A1: DS1.SC/DS1.BLK	A1
		DS2	DS2.SC/DS2.BLK	B1

Data Out	Description	Data In	Formula	Well
Lifetime	Transformation	DS1 DS2	40/LN(DS1/DS2)	Use single formula for all wells
Mean Lifetime	Transformation	Lifetime	MEAN(WELLID)	Use single formula for all wells
Mean OCR (OCR Must Be Entered First)	Transformation	OCR	MEAN(WELLID)	Use single formula for all wells

Data Out	Description	Data In	Calculation Type	Options
OCR	Kinetic analysis	Lifetime	Max V	10 minutes to >2 hours ~10 to 20 points RFU per hour

Data Out	Description	Data In	Curve Fit	Data Out
Cell optimization	Standard curve	STDC (from plate map) X-axis: concentration dilution Y-axis data: OCR	Point-to-point	Interpolations: Y-value 4 Y-value 5

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Published in the USA, August 21, 2020
5994-2322EN

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