

Optimization of the Agilent MitoXpress Intra Intracellular Oxygen Assay Using Agilent BioTek Gen5 Microplate Reader and Imager Software

Step-by-step instructions

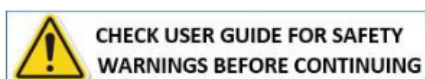
Gen5™



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The Agilent MitoXpress Intra intracellular oxygen assay allows real-time measurement of intracellular oxygen levels in 2D or 3D cultures. MitoXpress Intra allows for modeling oxygen concentrations of specific tissues or microenvironments on a plate reader. Agilent offers a dedicated filter cube assembly, preprogrammed Agilent BioTek Gen5 protocols, and a user tutorial tailored for Agilent BioTek instruments equipped with time-resolved fluorescence (TRF) capability.



Caution: This guide contains step-by-step instructions to maximize the functionality of the MitoXpress Intra protocol template (AgilentintraO2.prt) for Agilent BioTek Gen5 microplate reader and imager software. This guide includes plate reader setup, fluorophore loading, and signal optimization, as well as running the intracellular oxygen assay and cell monitoring assay, and data analysis. For detailed instructions on product use, please see the MitoXpress Intra User Guide.¹

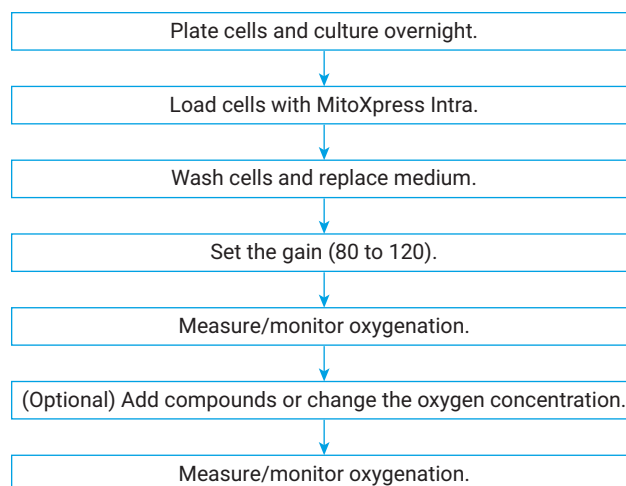


Figure 1. Workflow for the Agilent MitoXpress Intra Intracellular Oxygen Assay using an Agilent BioTek plate reader.

1. Instrument setup

Key steps

- 1.1 Cube installation
- 1.2 Protocol download
- 1.3 Loading MitoXpress Intra
- 1.4 Signal optimization

Key terms

Protocol: predefined procedure settings for Gen 5; can be edited for each experiment.

Experiment: output file containing data generated from a corresponding protocol

1.1 Cube installation

For use with Agilent BioTek Synergy H1 multimode reader or Agilent BioTek Cytation 1/5 cell imaging multimode readers, a filter cube is required. The Agilent BioTek MitoXpress filter cube (part number 8040587 Synergy H1 and Cytation)² or the Agilent BioTek MitoXpress pH-Xtra Glycolysis combination cube (part number 8040594 Synergy H1 and Cytation)³ can be used.

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 to the Optics Library, click **System > Optics Library > Filter Cubes**. Then, click **Add** and type a name for the filter cube. This name becomes available for selection in the Procedure section for Protocols and Experiments.

1.2 Protocol download

Download the AgilentIntraO2.prt Gen5 file

Location: [Gen5 Sample Files](#)

The template can be loaded in the Task Manager by clicking **Experiment > Create using an existing protocol or Protocols**.

Tip: for ease of use and reference in future experiments, save protocols as independent files anytime a modification has been made. Modifications to the procedure results in current data being replaced to preserve the audit trail.

Filter Cube

Name: MitoX-pH Xtra

☐ Fluorescence Polarization Cube

Filter Set 1

Filter Set Name: AgilentXO2

Excitation: Band Pass, 380, 20

Mirror: Dichroic, Top 400 nm, 400 nm

Emission: Band Pass, 645, 15

Get current values

Figure 2. Example of entering values for an oxygen filter cube into Optics Library.

Materials required

- MitoXpress Intra Intracellular Oxygen Assay (part number MX-300-4)
- Synergy H1 or Cytation 1 or Cytation 5
- CO₂ and O₂ gas controller (part number 1210013)
- Agilent Cell culture/imaging microplate, 96-well (part number 204626-100)
- DMEM: F-12 medium (ATCC part number 30-2006)

1.3 Loading MitoXpress Intra (adherent cells)

1. Plate cells following trypsinization (e.g., 40,000 to 65,000 cells/well) in growth medium, and allow to attach at room temperature before placing the plate in the incubator.
2. Culture cells overnight (typically >14 hours).
3. Resuspend MitoXpress Intra in 1.5 mL of media.
4. Add MitoXpress Intra to the cells at 1:10, i.e. 15 µL MitoXpress Intra in 150 µL media.
5. Return the plate to the CO₂ incubator and culture overnight.

On the day of the experiment:

6. Remove the media and wash the cells with media.
7. Add 150 µL of fresh media to each well.

1.4 Signal optimization

MitoXpress Intra accumulates inside or around the cells. To ensure that the instrument can detect the probe reliably, it is necessary to optimize the gain settings, this usually only needs to be done once for a given cell type. These instructions briefly describe how to test the signal-to-blank ratio (S:B) by editing the AgilentIntraO2 protocol. See the MitoXpress Intra User Guide¹ for complete instructions.

After loading MitoXpress Intra into the cells and washing, the gain setting should be adjusted so that the S:B is at least 3 and the signal should be stable after temperature equilibration (Figure 3). This means the TRF signal in each measurement window, from wells containing cells loaded with MitoXpress Intra, should be at least three times greater than that from wells with no MitoXpress Intra ("blank").

At ambient oxygen concentrations, using ~20,000 RFU for Read: 30_30 and ~5,000 RFU for Read: 70_30 (or maximum 20% Scale Percent) is a good starting value.

The optimal gain should allow for good starting intensity with a large enough window to measure increases in fluorescence without saturating the PMT.

Gain optimization is easily achieved using the Auto Gain function or can be adjusted manually. For manual adjustment, the gain is usually between 80 to 120.

1. In the Gen5 Task Manager, click **New Task > Experiments > Create** using existing protocol and select AgilentIntraO2.
2. Edit the procedure.
 - a. Select the correct plate type.
 - b. Edit the kinetic to read for 30 minutes.
 - c. Edit the read area of the Read: 30_30 to include the wells containing the blank and the MitoXpress Intra control (e.g. untreated cell loaded with MitoXpress Intra).
3. Click **Validate**.

Note: Auto Gain is selected as default. If you wish to change more settings, see Appendix.

4. Click **Create experiment > Read New**.
5. To set the Auto Gain, select two to four wells containing cells loaded with MitoXpress Intra.
6. Run the assay for 30 minutes and check that the average intensity is ~20,000 RFU in the Read: 30_30 user-entry field.
7. Save the protocol for future use, e.g., as "Optimized MX Intra".

Note: The instrument must be connected for the "Validate" function in Gen5. If validation fails, check that the read height is appropriate and adjust if necessary, or check that the minimum interval is sufficient.

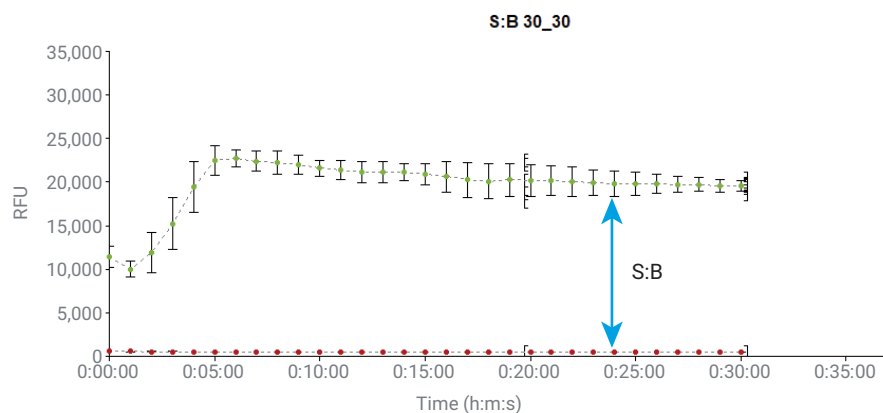


Figure 3. S:B ratio for 30_30 read. Mean values were calculated from 20 to 30 minutes. The S:B in this example was 40, which is typical for Agilent BioTek readers.

2. Intracellular Oxygen Assay

Key steps:

- 2.1 Plate preparation
- 2.2 Protocol setup
- 2.3 Oxygenation assay
- 2.4 Data reduction and results

Additional materials

- Agilent Seahorse XF Cell Mito Stress Test kit (part number 103015-100)
- A549 cells (ATCC, part number HB-8065)

The MitoXpress Intra Intracellular Oxygen Assay is a unique tool to quantitatively monitor the oxygen concentration that cells in culture are experiencing using an Agilent BioTek plate reader.

As cells respire, oxygen is consumed. This creates a microenvironment at the cellular level, where the oxygen concentration that the cells experience can be lower than the applied concentration (atmospheric or applied hypoxia). MitoXpress Intra is reversible so dynamic changes can be measured.

This experienced oxygen concentration can be influenced by cell metabolism and several other factors, including cell density, media composition, applied hypoxia, mitochondria modulation, and other microenvironmental changes, e.g., acidity. MitoXpress Intra enables the measurement of these oxygen concentrations at the cellular level, quantitatively.

Here we provide a step-by-step guide for monitoring cellular oxygenation, using a few examples for typical experimental setups and data outputs.

This section includes demonstrate how MitoXpress Intra can be used to:

- Measure changes in oxygenation when A549 cells are treated with mitochondrial modulators.
- Monitor the longer-term effects of cell density and growth on oxygenation using A549 cells.

2.1 Plate preparation

1. Plate 65,000 cells/well in complete media and allow to attach at room temperature before placing the plate in the incubator.

Note: Cell density optimization is recommended if this is the first experiment. Cell number will affect starting oxygenation levels (see the “Cell Monitoring Assay” section).

2. Culture cells overnight.
3. Resuspend MitoXpress Intra in 1.5 mL of media.
4. Add to the cells at 1:10, i.e. 15 μ L MitoXpress Intra in 150 μ L of media.
5. Return the plate to the CO₂ incubator and culture overnight.

On the day of the experiment:

6. Remove the media and wash cells once in media.
7. Add 150 μ L fresh media to each well.

Note: Blank wells are essential for lifetime and O₂ conversions. Ensure that blank wells are selected, i.e. wells with no cells and no probe. See Figure 4 for an example plate layout.

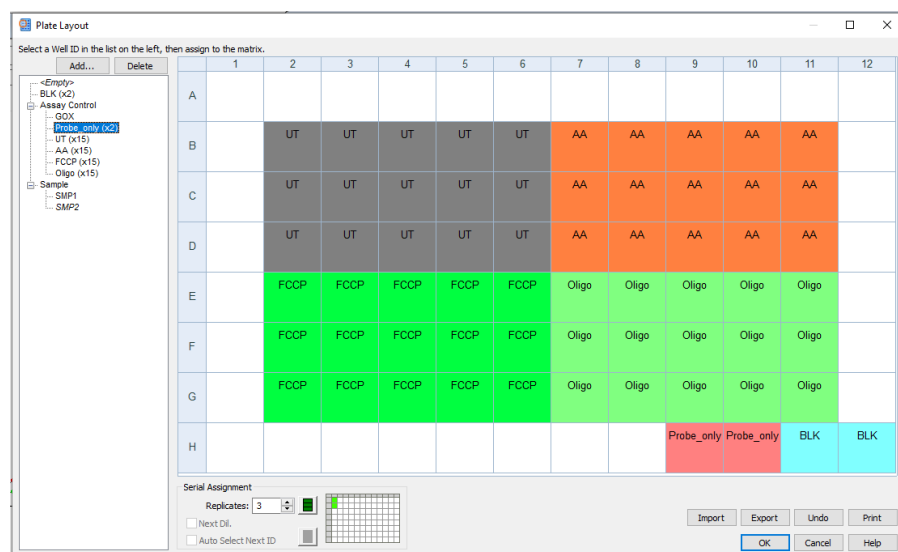


Figure 4. Typical plate layout.

2.2 Protocol setup

1. In the Gen5 Task Manager, click **New Task > Experiments > Create using existing protocol** and select the optimized protocol Optimized MX Intra.
2. Either check that Auto Gain is selected or use gain from the "Signal optimization" step.
3. In Procedure, edit the kinetic loop to run for 45 minutes at 2-minute intervals.
4. Add a Pause step.
 - a. Click **Plate Out/In**.
 - b. Comment "Add Compounds".
5. Copy the kinetic loop and paste it after the pause and change the time to 1 hour 15 minutes.
6. In both Read steps, select **Append to previous Kinetic data** (Figure 5).
7. Click **Validate**.
8. Select the wells and drag to populate.

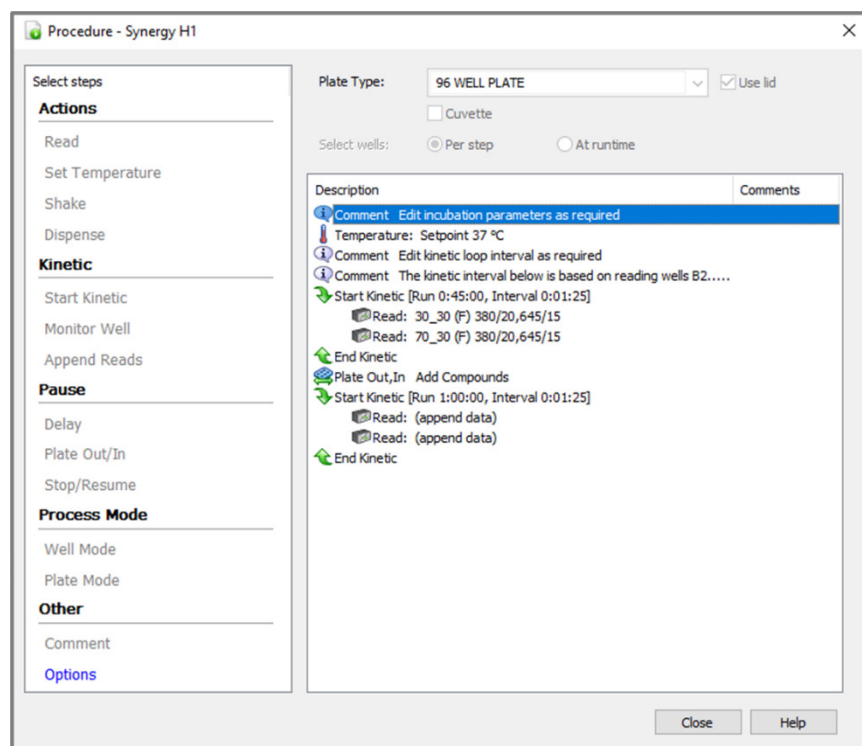


Figure 5. Procedure steps for an intracellular oxygenation assay with a pause step to add compounds following basal reads.

2.3 Oxygenation assay

1. Place the plate into the reader and allow to equilibrate for 30 minutes.
2. Start the assay.
3. Click **Read New**.
4. If using Auto Gain, select MitoXpress Intra loaded control cells to set the gain to 20% of the total.
5. While the plate is running, prepare compounds for addition (see Table 1).

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

6. Add the compounds to the microplate and restart the read when prompted by Gen5.

2.4 Data reduction and results

The Gen5 data reduction tool generates the signal change curves and converts the intensity data to lifetime and % O₂ based on a predetermined calibration function. Data can then be easily exported to graphing software for display and further analysis. The default calibration function to convert from lifetime to % O₂ was generated by applying several O₂ concentrations (21 to 1%) to nonrespiring cells using the gas controller, then applying a fit to lifetime versus applied O₂ graph (see Appendix for details).

In the plate window matrix, select **PercentIntaO2**. These values are the oxygen concentration the cells are experiencing. By selecting wells in **Curves [PercentIntaO2]**, signal trace curves can be displayed in a separate window, as in Figure 6. If the procedure has been set up as described, Gen5 displays the pre- and post-treatment groups in a single kinetic trace.

In this example, the untreated cells and all groups prior to compound addition had an oxygenation level of ~16%, demonstrating that, even under basal conditions, cells experience lower O₂ than what is being applied; in this case ~21%. The difference between applied and experienced O₂ is influenced by several factors, including cell type, respiration state, substrate availability, and cell density.

Note: the optimal FCCP concentration was determined previously using the MitoXpress Xtra Oxygen Consumption Assay.

The addition of FCCP (2.5 µM) caused the oxygenation levels to rapidly decrease to >5% O₂ (Figure 6, blue line). FCCP treatment uncouples the mitochondria, which increases oxygen consumption, leading to cells to experience lower oxygen levels compared to the untreated controls.

The addition of rotenone/antimycin A and oligomycin increases the oxygenation levels close to 21%; this is due to a decrease in oxygen consumption, as they are known inhibitors of the electron transport chain and ATP synthase.

Monitoring cellular oxygenation in real time using MitoXpress Intra allows researchers to accurately determine the depth of hypoxia experienced by a cell model or drug treatment. The assay enables researchers to relate metabolic responses to available oxygen levels. These parameters are important in areas such as ischemia, cancer metabolism, and hypoxia.

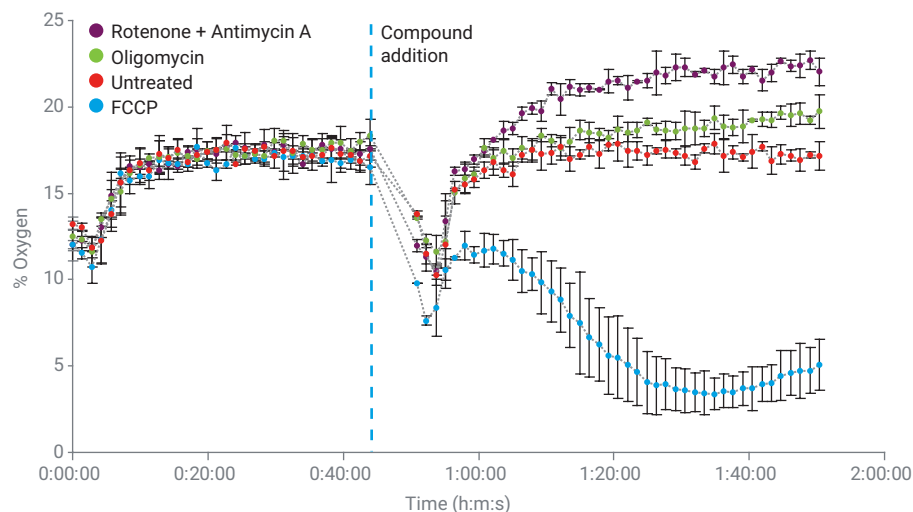


Figure 6. Oxygenation assay using Agilent MitoXpress Intra and compounds from an Agilent Seahorse XF Mito Stress Test. Oxygenation change is reflective of oxygen consumption rates in A549 cells when treated with mitochondrial modulators.

3. Cell monitoring assay

Key steps:

- 3.1 Assay setup
- 3.2 Protocol setup
- 3.3 Data reduction and results

Note: See the Manual⁴ for gas controller setup.

Note: For longer-term assays, water can be added to the outer wells and the spaces between wells of the 96-well microplate to maintain humidity.

One of the advantages of MitoXpress Intra is that the probe is stable for several days when loaded in cells. Using an Agilent BioTek gas controller with the Synergy H1 or Cytation 1/5 models, cells can be maintained at 37 °C, 5% CO₂ while being measured. MitoXpress Intra can be used to show how cells change the oxygen concentration of the microenvironment while they proliferate.

For most adherent cells, the recommended seeding densities are between 40,000 to 80,000 cells per well. However, as described above, cell type, substrate availability, respiration state, and applied oxygen concentration will also influence the starting oxygen concentration.

Here, we demonstrate the effect of seeding density using A549 cells in complete medium. Cells were measured for 48 hours.

3.1 Assay setup

1. Plate cells across a range of densities (40,000 to 80,000 cells/well) in growth media, and allow to attach at room temperature before placing the plate in the incubator.
2. Culture cells overnight.
3. On day 2, add MitoXpress Intra to wells (15 µL per well) and incubate for 12 hours.
4. Remove media and wash cells once with fresh media, then add 150 µL fresh medium to each well.
5. Set the gas controller unit to 5% CO₂.
6. (Optional): add rotenone/antimycin A (inhibitor of mitochondrial respiration) to a group for a nonrespiring cell control.

3.2 Protocol setup

1. In the Gen5 Task Manager, click **New Task > Experiments > Create using existing protocol** and select the optimized protocol **Optimized MX Intra**.
2. In Procedure, edit the kinetic loop to run for 48 hours at 1-hour intervals
3. Enter a plate map, click **Plate Layout**, then add a standard curve by clicking **Add > Standard Curve**, then enter the values for the cell numbers (Figure 7).
4. Select the wells and drag to populate.
5. Click **Read New**.

Optional Auto Gain step: set gain to 20% (or 20,000 RFU) and select wells with 40,000 cells or wells treated with Rotenone+Antimycin A.

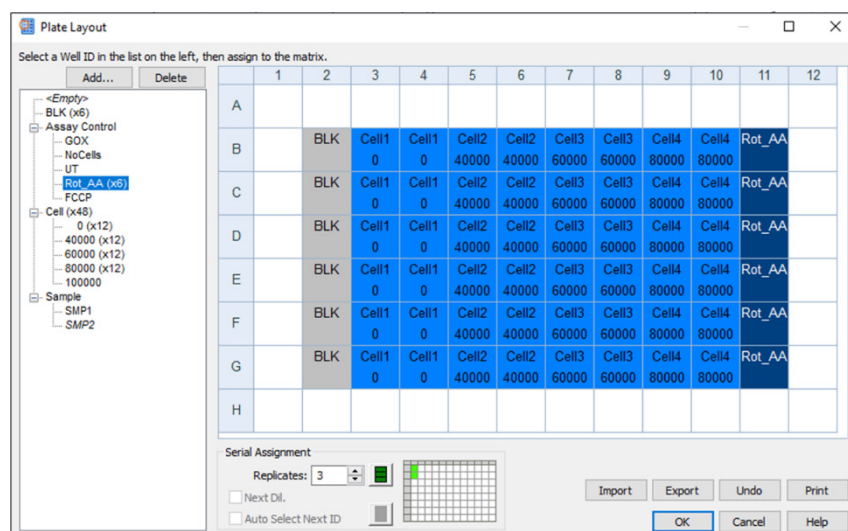


Figure 7. Example of a plate map used for typical longer-term assay; outer wells were filled with water to maintain humidity.

3.3 Data reduction and results

To visualise the titration, select PercentIntraO2 plot from the Graphs menu (Figure 8).

Cell number affects the starting oxygenation level. In this example, when 80,000 A549 cells/well were plated, the O_2 concentration that the cells initially experienced was around ~6%, compared to ~17% at 40,000 cells/well (Figure 8). As the cells proliferate, the increased demand of oxygen drives the cells into hypoxia. Note how the oxygen decreases to below 5% in the high-density cells after 12 hours (Figure 8, red line).

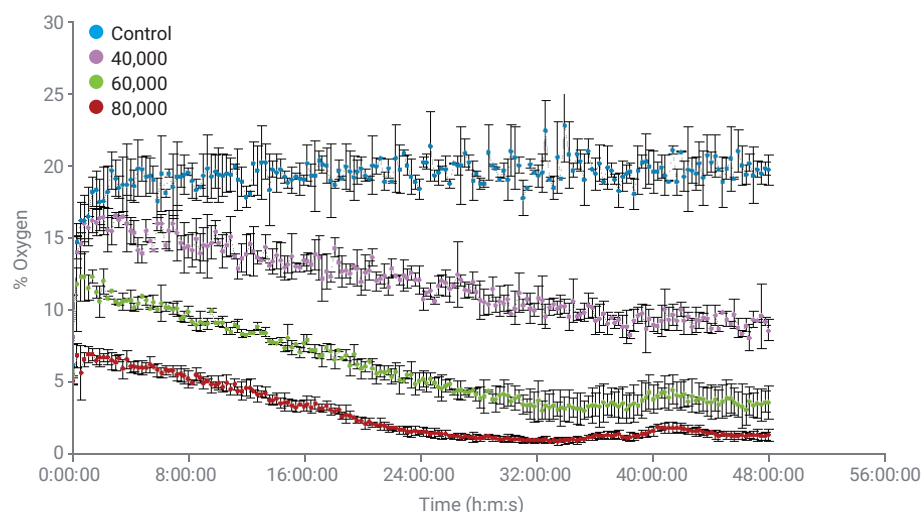


Figure 8. Longer-term cell-monitoring experiment showing the effects of initial cell density and proliferation on oxygenation.

Oxygen availability at the cellular level has a significant influence on cell physiology, signal transduction, and metabolism. As a result, more physiologically relevant oxygen concentrations are being used in in vitro studies, especially in cancer metabolism, drug discovery, neuronal, and cardiovascular research.

Understanding the depth and duration of the oxygenation condition experienced by the cell model is therefore of significant importance. This reduction in oxygenation can have significant physiological consequences, and if not measured, can lead to misinterpretation or inconsistency in data due to the lack of visibility of the oxygenation levels experienced by the cell model under study.

4. References

1. Agilent MitoXpress Intra Intracellular Oxygen Assay, User Guide 5991-9327
2. 8040587 MitoXpress filter cube for Synergy H1 & Cytation
3. 8040594 MitoXpress & pH-Xtra Combination filter for Synergy H1 & Cytation
4. Agilent Biotek Gas Controller Manual

5. Appendix

5.1 Calibration

To convert from lifetime to oxygen scale, a default calibration is provided with the protocol file, which should work in most conditions.

Default lifetime to % O₂ conversion:

$$\% \text{ O}_2 = 659.3 \cdot (\text{POW}(2.718(-1 \cdot x) / 8.475))$$

Calibration of the MitoXpress Intra probe was performed directly in live cells on the Agilent BioTek reader with a gas controller. The cells were treated with rotenone and antimycin A to inhibit respiration and ensure that the intracellular O₂ levels are equal to the external O₂ determined by the settings of the gas controller. Following ~30 minutes of temperature and gas equilibration of the plate at the selected % O₂, lifetime readings were taken in cells treated with inhibitors. Since it is difficult to equilibrate below 1% O₂, a zero O₂ calibration point was produced by chemical deoxygenation with glucose oxidase enzyme (0.1 mg/mL). Corresponding lifetime values were calculated and plotted against O₂ concentration in the medium at given applied % O₂. The resulting set of data points was fitted with a mathematical function ($R^2 > 0.9$), which was subsequently used as calibration function to convert measured lifetime values into intracellular O₂ concentration. This can serve as a reference for generating conversion functions if the default appears to be inaccurate; this can occur in certain media types.

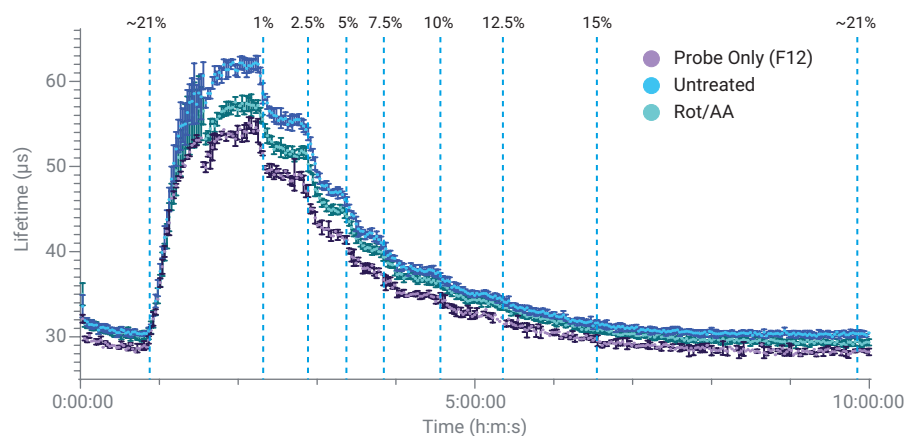


Figure 9. Calibration curve for O₂ using Agilent MitoXpress Intra and an Agilent BioTek gas controller.

5.2 Data reduction quick reference

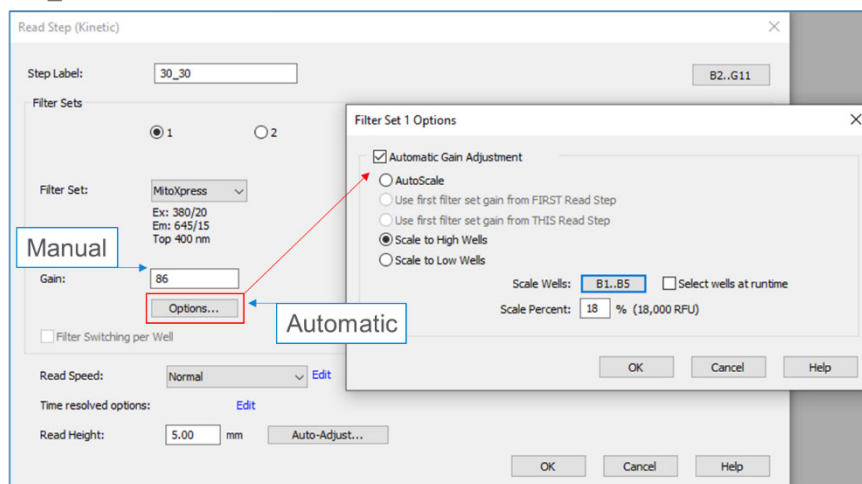
Data Out	Description	Data In	Formula	Well
S:B	Transformation	DS1	A1: DS1.SC/DS1.BLK	A1
		DS2	DS2.SC/DS2.BLK	B1
Data Out	Description	Data In	Formula	Well
Lifetime	Transformation	DS1 DS2	40/LN(DS1/DS2)	Use single formula for all wells
Mean Lifetime	Transformation	Lifetime	MEAN(WELLID)	Use single formula for all wells
% O ₂	Transformation	Lifetime	659.3 · (POW(2.718281845904 (-1 · X) / 8.475))	Use single formula for all wells

5.3 Changing the gain settings

Change the Read Step (Auto Gain)

1. Select **Read 30_30**.
2. Click **Options...**
3. Select **Automatic Gain Adjustment**.
4. Select **Scale to High Wells**.
5. Click **Scale Wells**, select wells with probe.
6. For Scale Percent: type 20.
7. Click **OK**.
8. Select **Read 70_30**.
9. Click **Options** and select **Use first filter set gain from FIRST Read Step**.

30_30



70_30

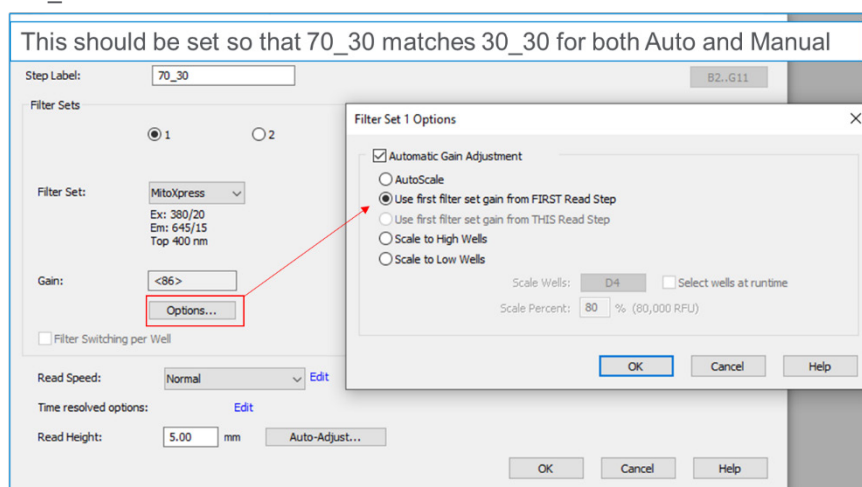


Figure 12. Gain setting for Agilent BioTek Gen5 microplate reader and imager software.

Change the Read Step (Manual Gain)

Note: gain is usually around 80 to 100.

1. Select **Read 30_30**.
2. Click **Options**.
3. Deselect **Auto Gain Adjustment**.
4. In Gain: type the gain value.

Note: gain is usually around 80 to 110.

5. Click **OK**.
6. Select **Read 70_30**.
7. Click **Options** then select **Use first filter set gain from FIRST Read Step**.

Note: Gain settings must match. If gain is adjusted on the 30_30 read, ensure that 70_30 is the same.

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RA44887.4207175926

© Agilent Technologies, Inc. 2022
Published in the USA, November 22, 2022
5994-4977EN