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Agilent MitoXpress Intra

Intracellular Oxygen Assay

For the measurement of Intracellular Oxygen Concentration

For use with:

- Cell lines
- Stem-cell derived cell types
- Primary cells
- 2D cultures, 3D cultures, and spheroids

Notices

Manual Part Number

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Contents

1 General Information

Materials Supplied

Assay kit will arrive at room temperature. For best results store as indicated below.

Cat No.	Item	96 well Quantity / Size	Storage
MX-300-4	MitoXpress Intra reagent	4 vials	+4 °C

Storage and Stability

The MitoXpress Intra Xtra reagent should be stored as follows:

- Dry material between +2 to +8 °C (see Exp. Date on vial).
- Product reconstituted in sterile water can be stored in the dark between +2 to +8 °C, for use within 3 weeks (DO NOT FREEZE).

Additional Items Required

- Fluorescence plate reader, with suitable filter(s) and plate temperature control.
- Standard clear 96-well TC$^+$ plates OR 96-well black wall clear bottom TC$^+$ plates.

Optional Items Not Supplied

- Plate block heater for plate preparation
- Controls: Antimycin A, FCCP, Glucose Oxidase
- Atmospheric Control Module or Workstation to control CO_2 / O_2 environment

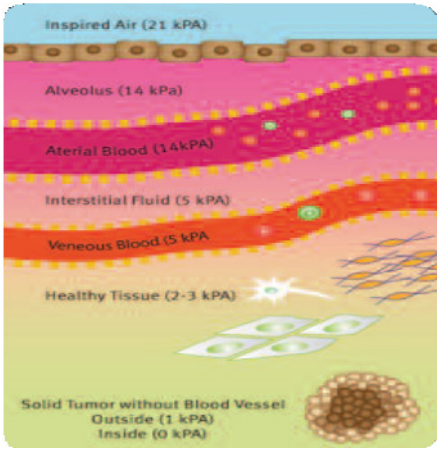
Support

Visit our website **www.agilent.com**.

2 Description

Oxygen availability significantly influences cell physiology, signal transduction and cellular response to drug treatment. However, in the majority of *in vitro* studies, cells are cultured at ambient oxygen despite the fact that it represents a hyperoxic state for most cell types. The assumption that cells experience this ambient condition also ignores the significant oxygen gradient that can exist between the atmosphere and respiring cells.

The MitoXpress Intra - Intracellular Oxygen Assay facilitates the convenient investigation and quantification of intracellular oxygen concentration (oxygenation), The assay is based on a proprietary O_2-sensitive cell-penetrating nanoparticle probe. The MitoXpress-Intra fluorophore, is chemically stable and inert, and is taken up by cells during an overnight loading period. Oxygen quenches the phosphorescent emission of the probe, such that measured signal (Ex/Em: 380 nm/650 nm) is proportional to intracellular oxygen concentration ($[iO_2]$), thereby allowing real-time monitoring of intracellular oxygen concentration in conventional 2D culture as well as a wide range of 3D systems, including Matrigel, RAFT, microtissues, Alvetex, Mimetix and other scaffold systems.



MitoXpress Intra provides a unique tool to quantitatively monitor the oxygen concentration that cells in culture are ACTUALLY experiencing. Additionally, where the experimental objective is to monitor cell physiology under defined O_2 conditions, MitoXpress Intra provides the ideal tool to identity the appropriate environmental O_2 to achieve this desired cellular O_2 concentration as it accounts for the significant impact cell respiration can have on intracellular O_2 concentration. The flexible plate reader format also allows multiparametric or multiplex combinations with other commonly used reagents.

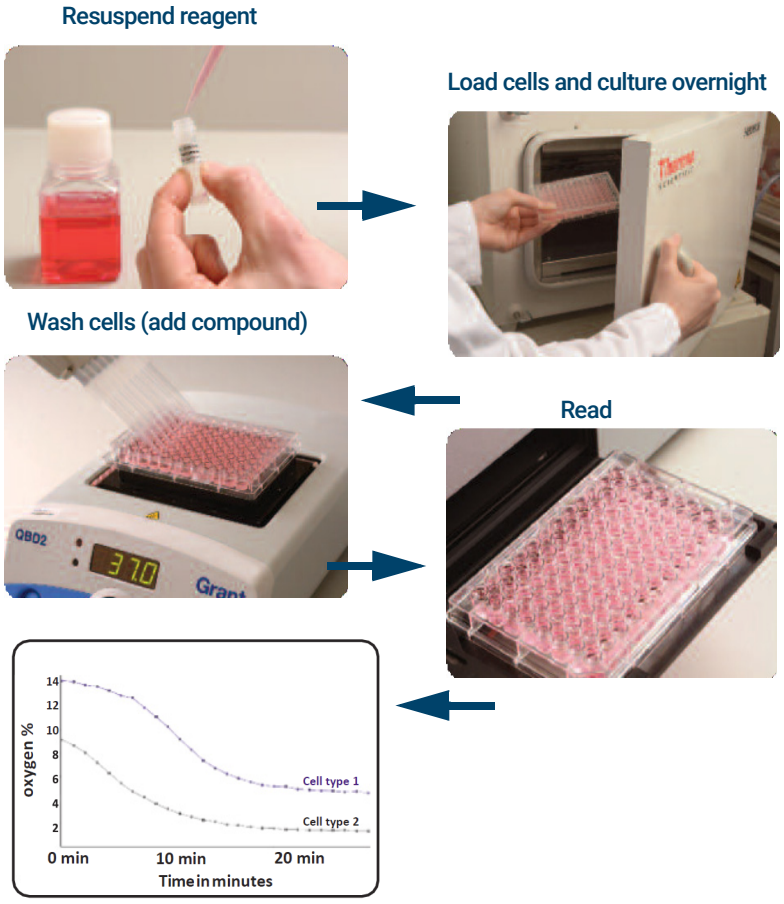


Figure 1. Flow diagram showing preparation and use of MitoXpress Intra - Intracellular Oxygen Assay

3 Plate Reader Set-Up

Measurement Parameters

MitoXpress Intra reagent is a chemically stable and inert, nanoparticulate, oxygen-sensing fluorophore.

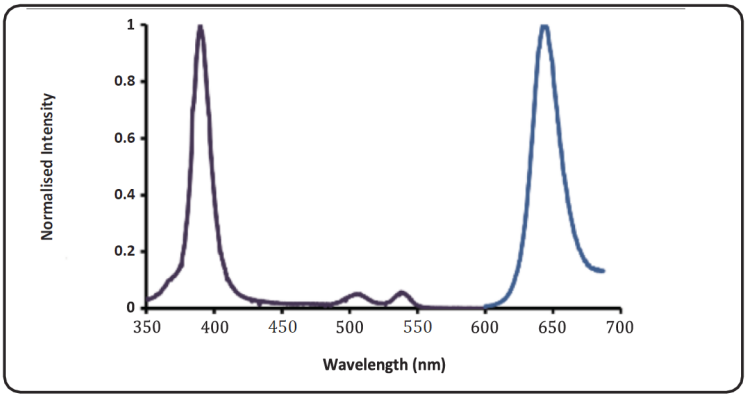


Figure 2. Excitation and Emission spectra of MitoXpress Intra, showing normalized excitation (Ex 360-400 nm; Peak 380 nm) and emission (Em 630-670 nm; Peak 650 nm).

Instruments and Settings

Two fluorescence modes can be successfully used with the MitoXpress-Intra Intracellular Oxygen Assay.

- **Standard:** Time-resolved fluorescence measurement (TR-F)
- **Advanced:** Dual-read Ratiometric TR-F measurement (Lifetime calculation)

NOTE

MitoXpress Intra - Intracellular Oxygen Assay may also be used semi-quantitatively in non-TR-F intensity mode, although we recommend running the described Signal Optimization protocol and optimizing cell seeding density.

NOTE

Further details, including instrument, filter selection and measurement settings can be found in "Appendix A - Instrument Settings" on page 27.

Signal Optimization - recommended for first time users

NOTE **Use a plate block heater for plate preparation and prewarm plate reader to measurement temperature.**

STEP 1: Reconstitute contents of a MitoXpress Intra reagent vial in 1 mL of sterile water, gently aspirating 3-4 times.

NOTE **Reconstituted stock can be stored in the dark between +2 to +4 °C for up to three weeks (DO NOT FREEZE).**

STEP 2: Prepare eight wells of a 96-well plate, by adding 150 µL prewarmed culture medium to each well (A1- A4, B1-B4).

STEP 3: Add 2.5 µL of reconstituted MitoXpress Intra reagent to four replicate wells (A1-A4) and 2.5 µL culture medium to the remaining replicate wells (B1-B4).

STEP 4: Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).

STEP 5: Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) signals and calculate signal- to-blank (S:B) ratio using the 30 minute read values.

NOTE **For dual read TR-F, calculate S:B for each measurement window.**

For most fluorescence TR-F plate readers, set up according to “Appendix A - Instrument Settings” on **page 27**, MitoXpress Intra reagent should return a S:B ≥ 8. See “Appendix D - Troubleshooting” on **page 33**.

	1	2	3	4
A	Media + MitoXpress Intra	Media + MitoXpress Intra	Media + MitoXpress Intra	Media + MitoXpress Intra
B	Media	Media	Media	Media

4 Performing the Intracellular Oxygen Assay

Cell Culture and Plating

NOTE **Always leave two wells (H11 and H12) free from the addition of MitoXpress Intra reagent, as Blank Controls.**

STEP 1: Count cells and adjust to the desired plating density in culture medium, (typically 30,000 - 80,000 cells per well for 2D cultures, see “Appendix C - Explanatory Notes” on **page 31** for information on 3D plating). To eliminate possible plate effects relating to evaporation and cell growth we recommend adding 200 µL of culture medium only (without cells) to the outer wells of 96-well plate (columns 1 and 12, rows A and H). Return the plate to culture overnight (typically > 14 hours).

MitoXpress Intra Reagent Loading

STEP 2: Reconstitute the contents of a MitoXpress Intra vial in 1 mL of sterile water aspirating 3-4 times. Dilute 1 in 10 in culture media to provide sufficient volume to load the required number of wells at 150 µL per well, then warm to measurement temperature (typically 37 °C).

STEP 3: After overnight culture (Step 1), place the 96-well plate containing cells on the plate block heater equilibrated at measurement temperature. Remove spent media (~200 µL) from each well using an aspirator being careful not to dislodge cells from the base of the wells.

NOTE **Do not allow cells to dry out during aspiration.**

STEP 4: Using a multichannel or repeater pipette add 100 µL of the prewarmed MitoXpress Intra stock (from Step 2), to each well being careful not to dislodge cells and return the plate to culture overnight culture (typically > 14 hours, see "Appendix C - Explanatory Notes" on **page 31** for alternative loading options).

Figure 3. Reconstitution of MitoXpress Intra reagent

Typical Assay

STEP 5: Place ~25 mL of culture media in a 50 mL plastic tube and warm to measurement temperature (37°C). For longer term measurements (>2 hours), we recommend the use of a HEPES buffer. See "Appendix C - Explanatory Notes" on **page 31** for more information.

STEP 6: Warm instrument to 37 °C and prepare a kinetic measurement protocol with the correct instrument settings to read the desired wells at 2-3 minute intervals over the desired duration (see "Appendix A - Instrument Settings" on **page 27**).

STEP 7: With the plate on a plate block heater, wash cells by removing spent media using an aspirator, and using a multichannel or repeater pipette add 100 µL of the prewarmed measurement buffer to each well.

NOTE **Take care not to dislodge cells from the base of the wells.**

STEP 8: Repeat wash step and finally add 150 µL of prewarmed measurement buffer to each test well and to designated Blank wells. (The Blank wells are required for the proper Blank correction of the measured fluorescence signal.)

NOTE **Plate preparation time should be kept to a minimum.**

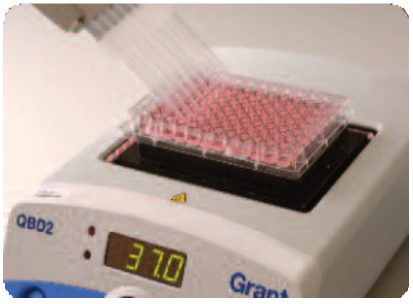


Figure 4. Removing loading media prior to measurement

STEP 9: Insert plate into the fluorescence plate reader and commence kinetic reading. Measure baseline signal for a minimum of 20 minutes to ensure sample temperature equilibration.

ADDING TEST COMPOUND: For manual compound addition, pause reading, eject the plate from the reader and quickly add test compound to each well. Re-insert the plate into the plate reader and recommence the kinetic measurement. If available, plate reader injectors can also be used. After responses are observed and oxygen levels have stabilized, further compound additions can be made.

MODULATING OXYGEN ENVIRONMENT: To assess the impact of altered ambient oxygen concentration using an atmospheric control module, after baseline signal has stabilized, alter ambient oxygen concentration as per manufacturer's instructions.

STEP 10: When measurement cycle is complete, remove plate from instrument and save data to file.

STEP 11: Plot the blank-corrected MitoXpress Intra Intensity or Lifetime values versus time. Results can be further transposed into O_2 scale as described in "Appendix B - Data Processing and Analysis" on **page 29**. Data analysis templates are available from some plate reader manufacturers, specifically configured to automate the analysis of the MitoXpress Intra - Intracellular Oxygen Assay.

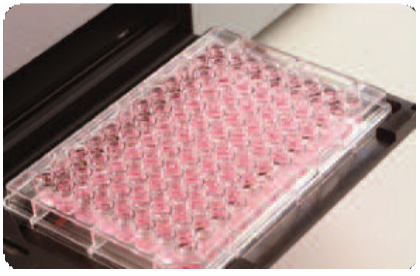


Figure 5. Reading the assay plate

Optional Controls

Signal Controls

Negative Signal Control: Leave two wells without cells for use as a Negative Signal Control. Add 150 µL of fresh culture media + 2.5 µL of reconstituted MitoXpress Intra reagent to each well (only for use when measuring in dual-read TR-F ratiometric mode).

Positive Signal Control: Leave two wells without cells for use as a Positive Signal Control. Add 150 µL of fresh culture media + 2.5 µL reconstituted MitoXpress Intra reagent and 10 µL of (1 mg/mL) Glucose Oxidase stock solution (prepared in water) to each well. (If measuring in intensity mode, add Glucose oxidase to wells containing MitoXpress Intra loaded cells).

Biological Controls

Negative Biological Controls: To two wells containing cells, add 1 µL of an Antimycin A stock solution (150 µM in DMSO). Antimycin blocks the electron transport chain (ETC) thereby removing the influence of cellular O_2 consumption on iO_2, typically seen as an increase in iO_2.

Positive Biological Control: To two wells containing cells, add FCCP to a final concentration of ~2 µM. FCCP uncouples respiration thereby increasing cellular O_2 consumption resulting in a decrease in iO_2.

NOTE **As FCCP exhibits a strong bel- shaped dose response, a serial dilution should be run of each new cell type to ensure the optimum concentration is used.**

5 Analysis

Data analysis typically uses MitoXpress Intra Lifetime or Intensity values plotted against time. Results can be further transposed into O_2 scale for further biological insight; data processing steps are described in detail in “Appendix B - Data Processing and Analysis” on **page 29**.

NOTE **We recommend that all first time users perform a Signal Optimization test, as described.**

Assessing Intracellular Oxygen Concentration

Data in O_2 scale can be generated using the conversions detailed in "Appendix B - Data Processing and Analysis" on **page 29**. Intracellular oxygen concentration can then be plotted against time to assess changes in cellular oxygenation allowing differences between ambient and intracellular O_2 to be determined. For example, HEK293T cultured in 2D and measured at ambient oxygen show an intracellular O_2 level of ~14% (**Figure 6**). Reducing instrument O_2 to 6% causes cellular oxygenation to drop to ~2% thereby quantifying the effect of respiratory activity on intracellular O_2.

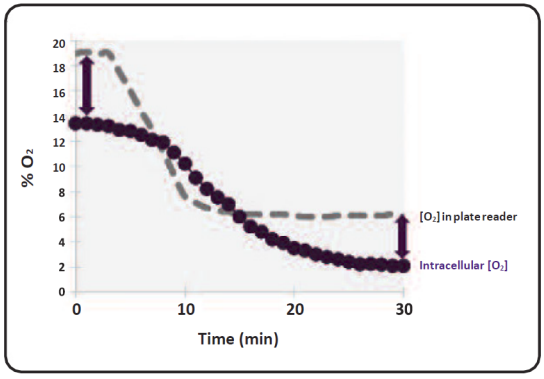


Figure 6. Measuring HEK293T cell oxygenation. Instrument O_2 and its impact on cellular oxygenation are presented.

Impact of Cell Metabolism on Oxygenation

Cellular oxygenation can be significantly affected by alterations in cell metabolism and these biological processes can be investigated in detail using the MitoXpress Intra - Intracellular Oxygen Assay. An example is presented in **Figure 7** whereby iPS derived cardiomyocytes measured at ambient O_2 (~21%), experience an intracellular O_2 of 14% under resting conditions. If the contribution of cellular respiration is removed through the addition of the electron transport chain (ETC) inhibitor Antimycin, intracellular O_2 slowly returns to ambient levels as the cells cease to consume O_2. If cardiomyocyte beat rate is increased through addition of the β-adrenoreceptor agonist isoproterenol, the resulting increase in ATP demand and O_2 consumption further reduces intracellular O_2 with values of ~6% observed for >15 minutes. This illustrates the significant O_2 depletion that cells can experience despite being measured at ambient O_2.

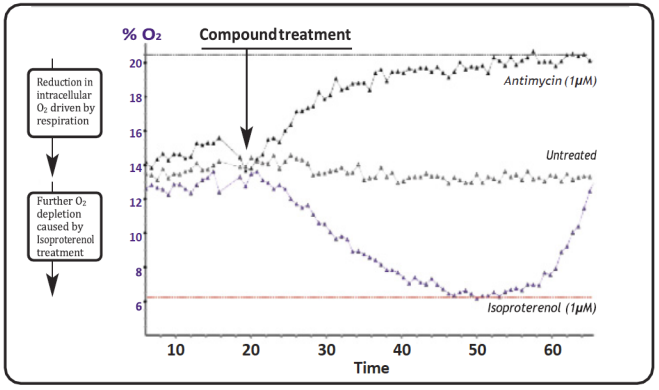


Figure 7. Measuring the impact of cell metabolism on iPS-derived cardiomyocyte oxygenation. During measurement, cells are treated with the ETC inhibitor Antimycin and the β-adrenoreceptor agonist isoproterenol.

Oxygen Scale Calibration

A default calibration function has been generated to facilitate the conversion of MitoXpress Intra Lifetime values into O_2 scale. This conversion is described in detail in "Appendix B - Data Processing and Analysis" on **page 29** and works well across multiple instruments and cell types. If required, a bespoke calibration function specific to a particular cell type, measurement temperature or measurement conditions can be generated. This requires access to a plate reader with an atmospheric control. An example is presented in **Figure 8**.

Cells are loaded with MitoXpress Intra reagent as described and, prior to measurement, treated with the ETC inhibitor Antimycin to remove the influence of cell respiration on intracellular O_2. Measurement begins at atmospheric O_2 (room air) and, using instrument atmospheric control, O_2 is reduced in a stepwise manner (typically ~20, 15, 10, 7.5, 5, 2.5, 1% O_2). Zero values are generated using glucose oxidase as described in the 'Optional Controls' section. Lifetime values are then plotted against applied [O_2] and a first order exponential fit applied to generate a calibration function (**Figure 8**).

NOTE **Calibrations are temperature specific and should be conducted at the desired measurement temperature.**

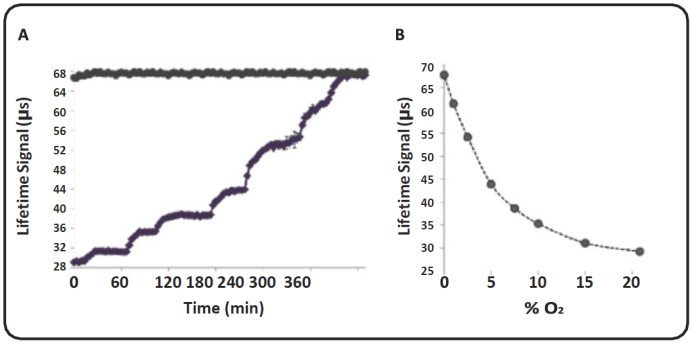


Figure 8. Sample Calibration Data. A) MitoXpress - Intra Lifetime profiles measured at decreasing [O_2] (purple line) with parallel glucose oxidase treatment to achieve 0% O_2 (grey line). B) The relationship between probe lifetime (T) and applied [O_2]. B) Applying a first order exponential fit generates a calibration function of O_2% = A1 x Exp(-T/ t1). Example: O2% = 659.3 x Exp(-T / 8.475). For additional detail see "Appendix B - Data Processing and Analysis" on **page 29**.

Appendix A - Instrument Settings

Two fluorescence modes can be successfully used to measure MitoXpress Intra reagent. Both use Time Resolved Fluorescence (TR-F) which reduces non-specific background and increases probe sensitivity. These measurement modes are outlined below and recommended instrument settings are listed in **Table 1** on page 28.

Standard: Single TR-F Measurement

Single TR-F measurement records probe signal after a defined delay, thereby reducing Blank signals and improving measurement performance. The optimal delay time is ~30 µs with a measurement window (integration time) of 100 µs.

NOTE

MitoXpress Intra should return a S:B > 8, values >10 are typical. Better S:B is achieved with filter-based optics.

Advanced: Dual-Read TR-F Measurement (Lifetime)

Optimal performance is achieved using dual-read TR-F (**Figure 9**) whereby two intensity measurements are taken sequentially. In combination with the Lifetime calculation detailed in "Appendix B - Data Processing and Analysis" on **page 29**, this ratiometric measurement approach monitors the rate of MitoXpress-Intra fluorescence decay, providing a more robust measurement of oxygen concentration. Optimal dual-delay and window times are: Read 1: 30 µs delay (D1), 30 µs measurement time (W1), Read 2: 70 µs delay (D2), 30 µs measurement time (W2).

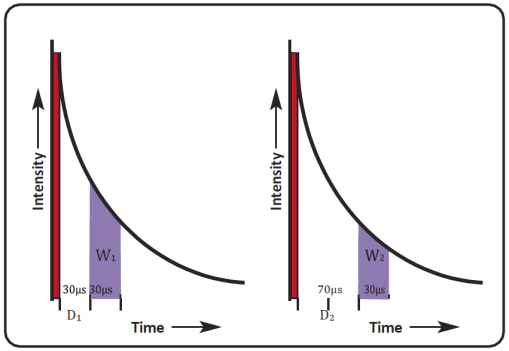


Figure 9. Illustrating dual-read TR-F measurement.

Table 1 Recommended Instrument and Measurement Settings

Instrument	Optical configuration	Integ 1 (D1 / W1) Integ 2 (D2 / W2)	Optimum mode	Ex (nm) Em (nm)
BioTek: Cytation 1, 3 or 5 Synergy H1	Filter-based Top or bottom read	30 / 30 µs 70 / 30 µs	Dual read TR-F (Lifetime)	Ex 380 ± 20 nm Em 645 ± 15 nm
BMG Labtech: CLARIOstar	Filter-based Top or bottom read (bottom read preferred where available)	30 / 30 µs 70 / 30 µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50 nm (TR-EX) Em 665 ± 50 nm or Em 645 ± 20 nm with LP-TR Dichroic
Tecan: Spark (10M/20M)	Filter-based Top read	30 / 30 µs 70 / 30 µs	Dual read TR-F (Lifetime)	Ex 380 ± 20 nm Em 670 ± 40 nm
	Fusion optics Top read	30 / 30 µs 70 / 30 µs	Dual read TR-F (Lifetime)	Ex 380 ± 20 nm (Monochromator) Em 670 ± 40 nm (Filter)

NOTE **Choose filter based optical configuration where available. Instruments with recommended Dual read TR-F measurement mode can alternatively be set up using standard TR-F measurement mode if desired.**

Appendix B - Data Processing and Analysis

The following description details how to manually calculate lifetime values from measured intensity values, and how to convert either lifetime or intensity data into oxygen scale. Specifically configured data analysis templates available from BMG Labtech and BioTek perform these calculations automatically.

Lifetime calculations from Dual-Read TR-F Measurements

Lifetime (T) values are calculated using the intensity data from Dual-Read TR-F Measurements as follows:

$$\tau = \frac{D_2 - D_1}{\ln(I_{W1}/I_{W2})}$$

where T represents emission lifetime and I_{W1} and I_{W2} represent signals measured at window 1 and window 2. Lifetime values are in µs units, (range ~29 to ~68 µs) and should only be calculated from samples containing MitoXpress Intra reagent. Lifetime values should not be calculated from Blank wells.

Converting into Oxygen Scale

Converting from Lifetime data

Lifetime data is favored as the basis for generating data in O_2 scale. Lifetime values as calculated above can be converted in oxygen scale using the following default analytical function:

$$O_2\% = 659.3 \times Exp\frac{-T}{8.475}$$

NOTE

This function is specific to measurements at 37 °C. Customized data analysis templates are available from some plate reader manufacturers, specifically configured to automate the analysis of the MitoXpress Intra - Intracellular Oxygen Assay. Bespoke analytical functions specific to particular cell types or measurement conditions can be generated if desired and require access to a plate reader with an atmospheric control capabilities. An example is presented in Figure 8 on page 26.

Converting from Intensity data

Intensity values can be converted into oxygen scale by including a positive control (glucose oxidase addition) and negative control (Antimycin addition). These controls are described in the optional controls section and should be generated on each test plate. Oxygen concentration ($[O_2]_t$) is then calculated using the following equation:

$$[O_2]_t = \frac{[O_2]_a \times I_a \times (I_0 - I_t)}{I_t \times (I_0 - I_a)}$$

whereby $[O_2]_a$ is oxygen concentration in air-saturated conditions (typically ~20.9%), I_0 and I_a are MitoXpress-Intra signals measured in deoxygenated and air-saturated conditions respectively, while I_t are the experimental intensity values generated during measurement.

NOTE

If the test plate has equilibrated in an environment containing 5% CO_2 and 95% humidity, ambient O_2 = ~18.6%.

Appendix C - Explanatory Notes

Cell Plating

Typically, cells are plated at a density to achieve full confluence. Plating density and basal metabolic rate will determine the steady-state oxygen concentration at the cell monolayer. When plating 3D cultures, prepare the 3D plate or 3D construct solution (e.g. collagen reaction mix) in advance as per manufacturer's instructions. Cells are typically plated at higher concentrations than used for 2D cultures. Lower plating concentration are typically used with long culture times unless cells are terminally differentiated.

Reagent Loading

If necessary, shorter incubation times can be used in combination with higher concentrations of MitoXpress Intra reagent. For example, a 6 hour reagent loading incubation using a 1.5X reagent concentration will yield comparable loading. This may be cell type dependent, so optimization is recommended if changes to reagent loading conditions are made. Reagent loading can also be performed directly in culture flasks if necessary.

Longer Term Measurements

A HEPES-based DMEM measurement buffer allows longer term measurement (>2 hours) outside a CO_2 incubator as it maintains pH for a prolonged period, without applied CO_2. This is not necessary if using plate reader models equipped with an atmospheric control module where 5% CO_2 can be maintained within the measurement chamber.

Appendix C - Explanatory Notes

Appendix D - Troubleshooting

Extensive literature, including Protocols, Application Notes, Videos, Publications and email technical support is also available through our website **www.agilent.com**.

General Notes and Recommendations

Storage and Stability: On receipt the MitoXpress Intra reagent should be stored between +2 to +8 °C (see Exp. Date on vial). Reconstituted reagent stock can be stored in the dark between +2 to +8 °C for 3 weeks **(DO NOT FREEZE)**.

Plate Reader: A fluorescence plate reader capable of measuring in TR-F mode, excitation at 380 nm and emission at 650 nm, and having plate temperature control is required.

Plates: We recommend 96 black wall / clear bottom TC+ plates, although standard clear wall polystyrene plates for cell culture may also be used. The assay is also compatible with many 3D culture plate systems.

Temperature: We recommend the use of a plate block heater for plate preparation steps, to maintain a temperature of 37 °C. Prewarm the fluorescence plate reader to measurement temperature and ensure that all culture media and stock solutions to be used in the assay are prewarmed at 37 °C prior to use.

Signal Optimization and Use of Controls: We recommend performing a Signal Optimization check, especially for first time users.

General Assay Set-Up, Pipetting and Aspirating: Prepare your assay, materials and work space in advance. Take care not to disrupt the cell monolayer (adherent cells) during pipetting and aspirating.

Cell Type and Cell Density: Since the MitoXpress Intra reagent requires cell loading, measured signal is dependent on both cell confluence and cell loading. Increased cell densities can also result in lower intracellular oxygen concentrations due to increased levels of cellular oxygen consumption.

Signal to Blank (S:B) Optimization

For most fluorescence plate readers, set up according to "Appendix A - Instrument Settings" on **page 27**, MitoXpress Intra should return a signal to blank ratio ≥ 8 Values of >10 are typical. The following options may be helpful to improve S:B if the determine ratio is not as high as expected:

1. Increase Gain (PMT) setting or flash energy.
2. Adjust TR-F focal height.
3. Repeat without phenol red or serum.
4. Repeat as top or bottom-read, respectively.
5. Increase the loading concentration of MitoXpress Intra.
6. Contact Instrument Supplier or **cellanalysis.support@agilent.com** for further options.

Frequently Asked Questions

Q: What do I do if I cannot detect any signal in wells containing cells and MitoXpress Intra (or I can detect a signal but the slope (rate) appears very low)?

A: Check correct Instrument Settings (“Appendix A - Instrument Settings” on **page 27**)- Perform Signal Optimization - Include Signal controls as described above - Increase cell density. If tested and not resolved, contact **www.agilent.com**.

Q: What do I do if I can detect a signal in wells containing MitoXpress Intra loaded cells, but the signal level falls initially or is variable from well to well?

A: Check cell seeding and pipetting consistency, increase cell density, ensure plate, instrument and all culture media and stock solutions are prewarmed at 37 °C prior to use, reduce plate preparation times.

NOTE

Some plate readers have inconsistent temperature control. If you suspect this to be the case, consider: Reducing assay (and equilibration) temperatures to 30 °C and avoid outer wells. If tested and not resolved, contact www.agilent.com.

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RELATED PRODUCTS

- pH-XtraTM - Glycolysis Assay (Cat No. PH-200-4)
- MitoXpress Xtra - Extracellular Oxygen Consumption (MX-200-4)

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