



Product Manual

MITO-ID[®] Intracellular O₂ Sensor Probe

Catalog #: ENZ-51046-K100



Product Manual

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Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION AND ASSAY PRINCIPLE

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.



To facilitate the convenient investigation and quantification of intracellular oxygen concentration (oxygenation), Enzo Life Sciences has developed the MITO-ID[®] Intracellular O₂ Sensor Probe - Intracellular Oxygen Assay, based on a proprietary O₂-sensitive cell-penetrating nanoparticle probe. The MITO-ID[®] Intracellular O₂ Sensor Probe 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: 380nm/650nm) is proportional to intracellular oxygen concentration ([iO₂]), 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.

MITO-ID[®] Intracellular O₂ Sensor Probe 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 O₂ conditions, MITO-ID[®] Intracellular O₂ Sensor Probe 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. Our flexible plate reader format also allows multiparametric or multiplex combinations with related Enzo Life Sciences products, as well as other commonly used reagents. For example, MITO-ID[®] Intracellular O_2 Sensor Probe in combination with the MITO-ID[®] Extracellular pH Sensor Probe (Cat. No. ENZ-51048) allows a detailed analysis of the interplay between O_2 availability and concomitant alterations in the balance between aerobic and glycolytic ATP production; investigations that are particularly relevant in the study of tumor metabolism.

KIT CONTENTS

MITO-ID[®] Intracellular O_2 Sensor Probe (solid form).

STORAGE AND STABILITY

The MITO-ID[®] Intracellular O_2 Sensor Probe should be stored as follows:

1. Dry material between 2°C to 8°C
2. Product reconstituted in sterile water can be stored in the dark between 2 °C to 8 °C, for use within 3 weeks (**DO NOT FREEZE**)

FORMAT

Easy “mix and measure” plate-based assay using standard 96-well plate. Total of 100 reactions are possible. Probe is a nanoparticulate metalloporphyrin-based, phosphorescent, oxygen sensitive probe.



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OTHER MATERIALS NEEDED

1. Fluorescence plate reader, with suitable filter and plate temperature control
2. 96-well (black wall) clear bottom TC+ plates or standard PS plates for cell culture
3. Plate block heater for plate preparation
4. Atmospheric Control Module or Workstation to control CO₂/O₂ environment

PRECAUTIONS

1. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
2. Interpretation of the results is the sole responsibility of the user.
3. Avoid freeze-thaw cycles.

WORKFLOW

Re-suspend reagent



Load cells and culture overnight



Wash cells (add compound)



Read



Figure 1: Flow diagram showing preparation and use of MITO-ID® Intracellular O₂ Sensor Probe.

METHODS AND PROCEDURES

PLATE READER SET-UP

Cell Culture Preparation

MITO-ID[®] Intracellular O₂ Sensor Probe reagent is a chemically stable and inert, nanoparticulate, oxygen-sensing fluorophore.

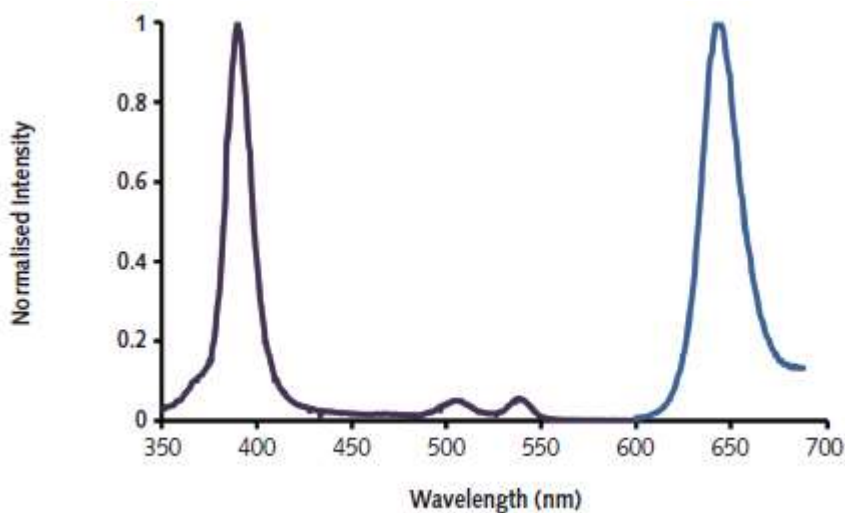


Figure 2: Excitation and Emission spectra of MITO-ID[®] Intracellular O₂ Sensor Probe, showing normalized excitation (Ex: 360 – 400 nm; Peak 380 nm) and emission (Em: 630- 670 nm; Peak 650 nm).

INSTRUMENTS AND SETTINGS

Two fluorescence modes can be successfully used with the MITO-ID[®] Intracellular O₂ Sensor Probe Intracellular Oxygen Assay.

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

NOTE: MITO-ID[®] Intracellular O₂ Sensor Probe - Intracellular Oxygen Assay may also be used semi-quantitatively in non-TR-F



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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.

SIGNAL OPTIMIZATION

This procedure is recommended for first time users.

NOTE: Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature.

1. Reconstitute contents of the MITO-ID[®] Intracellular O₂ Sensor Probe 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 3 weeks (DO NOT FREEZE).*
2. Prepare 8 wells of a 96-well plate, by adding 150 µL pre-warmed culture medium to each well (A1-A4, B1-B4).
3. Add 2.5 µL of reconstituted MITO-ID[®] Intracellular O₂ Sensor Probe reagent to 4 replicate wells (A1-A4) and 2.5ML culture medium to the remaining replicate wells (B1-B4).
4. Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).
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.*

NOTE: For most fluorescence TR-F plate readers, set up according to Appendix A - Instrument Settings, MITO-ID[®] Intracellular O₂

Sensor Probe reagent should return a S:B \geq 8. See Appendix D - Troubleshooting.

	1	2	3	4
A	Media + MITO-ID [®] Intracellular O ₂ Sensor Probe	Media + MITO-ID [®] Intracellular O ₂ Sensor Probe	Media + MITO-ID [®] Intracellular O ₂ Sensor Probe	Media + MITO-ID [®] Intracellular O ₂ Sensor Probe
B	Media	Media	Media	Media

PERFORMING THE INTRACELLULAR OXYGEN ASSAY

CELL CULTURE AND PLATING

NOTE: Always leave two wells (H11 and H12) free from the addition of MITO-ID[®] Intracellular O₂ Sensor Probe reagent, as Blank Controls.

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 for information on 3D plating). To eliminate possible plate effects relating to evaporation and cell growth we recommend adding 200 μ L 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 > 14h).

MITO-ID[®] INTRACELLULAR O₂ SENSOR PROBE REAGENT LOADING

2. Reconstitute the contents of the MITO-ID[®] Intracellular O₂ Sensor Probe 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 μ L per well, then warm to measurement temperature (typically 37 °C).

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 μ L) 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.*



Figure 3: Reconstitution of MITO-ID[®] Intracellular O₂ Sensor Probe Reagent

4. Using a multichannel or repeater pipette add 100 μ L of the pre-warmed MITO-ID[®] Intracellular O₂ Sensor Probe stock (from Step 2), to each well being careful not to dislodge cells and return the plate to culture overnight culture (typically > 14h, see Appendix C - Explanatory Notes for alternative loading options).

TYPICAL ASSAY

5. Place ~25 mL of culture media in a 50 mL plastic tube and warm to measurement temperature (37°C). For longer term measurements (>2h), we recommend the use of a HEPES buffer. See Appendix C - Explanatory Notes for more information.
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).
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 μ L of the prewarmed measurement buffer to each well.

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

8. Repeat wash step and finally add 150 μ L of pre-warmed 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.

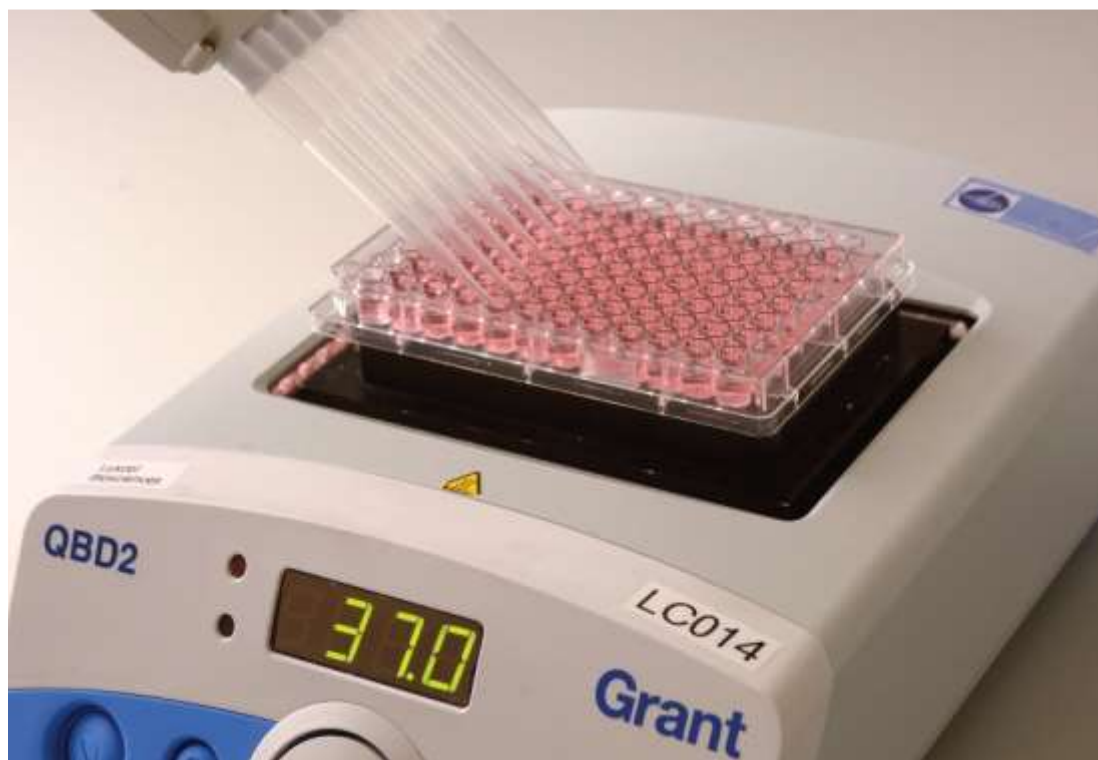


Figure 4: Removing loading media prior to measurement.

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.

- 10.** When measurement cycle is complete, remove plate from instrument and save data to file.
- 11.** Plot the blank-corrected MITO-ID[®] Intracellular O₂ Sensor Probe Intensity or Lifetime values versus time. Results can be further transposed into O₂ scale as described in detail Appendix B - Data Processing and Analysis. Data analysis templates are available from some plate reader manufacturers, specifically configured to automate the analysis of the MITO-ID[®] Intracellular O₂ Sensor Probe.

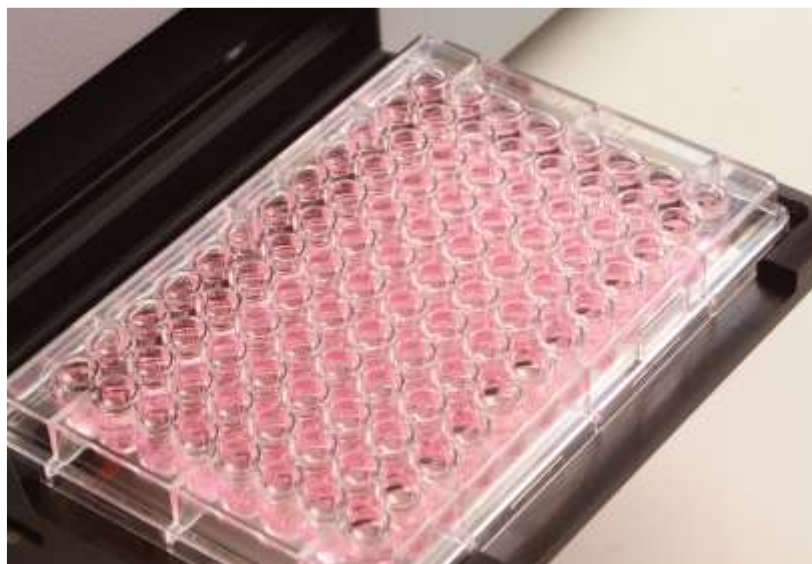


Figure 5: Reading the assay plate

OPTIONAL CONTROLS

SIGNAL CONTROLS:

Negative Signal Control: Leave 2 wells without cells for use as a Negative Signal Control. Add 150 μL of fresh culture media + 2.5 μL of reconstituted MITO-ID[®] Intracellular O₂ Sensor Probe reagent to each well (only for use when measuring in dual-read TR-F ratiometric mode).

Positive Signal Control: Leave 2 wells without cells for use as a Positive Signal Control. Add 150 μL of fresh culture media + 2.5 μL reconstituted MITO-ID[®] Intracellular O₂ Sensor Probe 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 MITO-ID[®] Intracellular O₂ Sensor Probe loaded cells).

BIOLOGICAL CONTROLS:

Negative Biological Controls: To 2 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 O₂ consumption on iO₂, typically seen as an increase in iO₂.

Positive Biological Control: To 2 wells containing cells, add FCCP to a final concentration of ~ 2 μM . FCCP uncouples respiration thereby increasing cellular O₂ consumption resulting in a decrease in iO₂.

NOTE: As FCCP exhibits a strong bell shaped dose response, a serial dilution should be run of each new cell type to ensure the optimal concentration is used.

ANALYSIS

Data analysis typically uses MITO-ID[®] Intracellular O₂ Sensor Probe Lifetime or Intensity values plotted against time. Results can be

further transposed into O₂ scale for further biological insight; data processing steps are described in detail in Appendix B: Data Processing and Analysis. Data analysis templates are available from BMG Labtech and BioTek, specifically configured to automate the analysis of the MITO-ID[®] Intracellular O₂ Sensor Probe with their plate readers. *NOTE: We recommend that all first time users perform a Signal Optimization test, as described.*

ASSESSING INTRACELLULAR OXYGEN CONCENTRATION

Data in O₂ scale can be generated using the conversions detailed in Appendix B - Data Processing and Analysis. Intracellular oxygen concentration can then be plotted against time to assess changes in cellular oxygenation allowing differences between ambient and intracellular O₂ to be determined. For example, HEK293T cultured in 2D and measured at ambient oxygen show an intracellular O₂ level of ~14% (Fig. 6). Reducing instrument O₂ to 6% causes cellular oxygenation to drop to ~2% thereby quantifying the effect of respiratory activity on intracellular O₂.

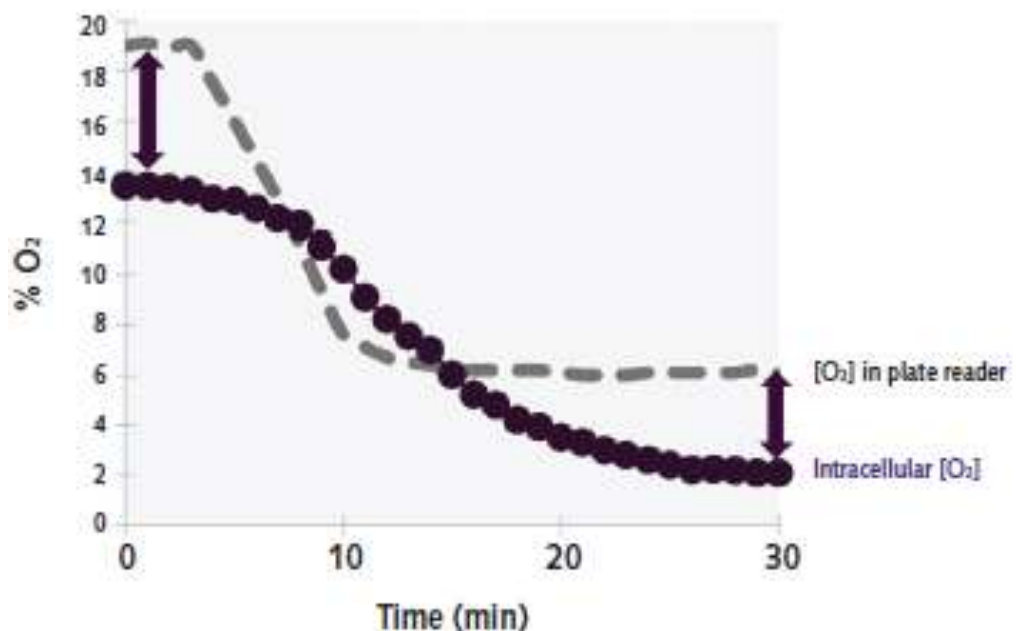


Figure 6: Measuring HEK293T cell oxygenation MITO-ID[®] Intracellular O₂ Sensor Probe measurement and O₂ modulations performed using a CLARIOstar equipped with an ACU module (BMG Labtech). Instrument O₂ (- -) and its impact on cellular oxygenation (...) are presented.

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 MITO-ID[®] Intracellular O₂ Sensor Probe. An example is presented in Fig 7 whereby iPS derived cardiomyocytes measured at ambient O₂ (~21%), experience an intracellular [O₂] 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 O₂ slowly returns to ambient levels as the cells cease to consume O₂. If cardiomyocyte beat rate is increased through addition of the β-adrenoceptor agonist isoproterenol, the resulting increase in ATP demand and O₂ consumption further reduces intracellular O₂, with values of ~6% observed for >15 min. This illustrates the significant O₂ depletion that cells can experience despite being measured at ambient O₂.

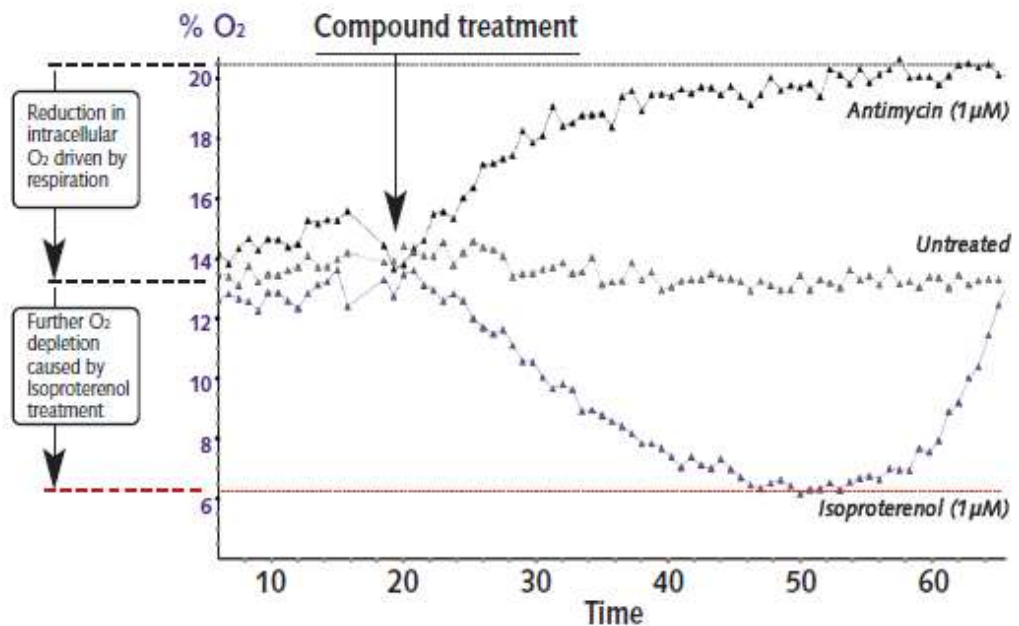


Figure 7: Measuring the impact of cell metabolism on iPS-derived cardiomyocyte oxygenation. During measurement, cells are treated with the ETC inhibitor Antimycin and the β-adrenoceptor agonist isoproterenol.

OXYGEN SCALE CALIBRATION

A default calibration function has been generated to facilitate the conversion of MITO-ID[®] Intracellular O₂ Sensor Probe Lifetime values into O₂ scale. This conversion is described in detail in Appendix B - Data Processing and Analysis 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 Fig 8.

Cells are loaded with MITO-ID[®] Intracellular O₂ Sensor Probe reagent as described and, prior to measurement, treated with the ETC inhibitor Antimycin to remove the influence of cell respiration on intracellular O₂. Measurement begins at atmospheric O₂ (room air) and, using instrument atmospheric control, O₂ is reduced in a stepwise manner (typically ~20, 15, 10, 7.5, 5, 2.5, 1% O₂). Zero values are generated using glucose oxidase as described in the 'Optional Controls' section. Lifetime values are then plotted against applied [O₂] and a first order exponential fit applied to generate a calibration function (Fig. 8).

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

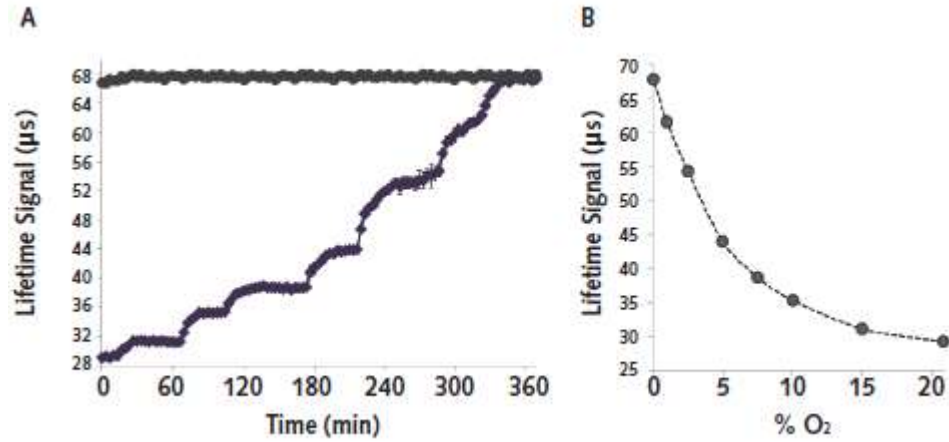


Figure 8: Sample Calibration Data. A) MITO-ID® Intracellular O₂ Sensor Probe Lifetime profiles measured at decreasing [O₂] (purple line) with parallel glucose oxidase treatment to achieve 0% O₂ (grey line). B) The relationship between probe lifetime (τ) and applied [O₂]. B) Applying a first order exponential fit generates a calibration function of $O_2\% = A1 \times \text{Exp}(-\tau / t1)$.

Example: $O_2\% = 659.3 \times \text{Exp}(-\tau / 8.475)$. For additional detail see Appendix B - Data Processing and Analysis.

APPENDIX

APPENDIX A - INSTRUMENT SETTINGS

Two fluorescence modes can be successfully used to measure MITO-ID[®] Intracellular O₂ Sensor Probe 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.

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: MITO-ID[®] Intracellular O₂ Sensor Probe 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 (Fig. 9) whereby two intensity measurements are taken sequentially. In combination with the Lifetime calculation detailed in Appendix B, this ratiometric measurement approach monitors the rate of MITO-ID[®] Intracellular O₂ Sensor Probe 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).

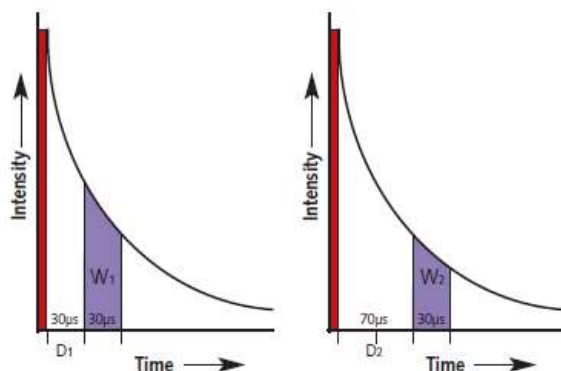


Figure 9: Illustrating dual-read TRF measurement.

TABLE 1: RECOMMENDED INSTRUMENT SETTINGS



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Instrument	Optical Configuration	Integ 1 (D1/W1) Integ 2 (D2/W2)	Optimum Mode*	Ex (nm) Em (nm)
BioTek: Cytation 3 / 5	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 380 \pm 20 nm Em: 645 \pm 15 nm
BioTek: Synergy H1 / Neo/ 2	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 380 \pm 20 nm Em: 645 \pm 15 nm
BMG Labtech: CLARIOstar	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 340 \pm 50 nm (TR-EX) Em: 665 \pm 50 nm or Em 645 \pm 10 nm
BMG Labtech: FLUOstar Omega / POLARstar Omega	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 340 nm \pm 50 nm (TR-EXL) Em: 655 nm \pm 25 nm (BP-655)
Perkin Elmer: VICTOR series/X4/ X5	Filter-based Top read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 340 \pm 40 nm (D340) Em: 642 \pm 10 nm (D642)
Tecan: Infinite M1000Pro /F200Pro	Monochromator Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 380 \pm 20 nm Em: 650 \pm 20 nm or Em: 670 \pm 40 nm
Tecan: Spark 10M/20M	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual read TR-F (Lifetime)	Ex: 380 \pm 20 nm Em: 650 \pm 20 nm or Em: 670 \pm 40 nm
BioTek: Synergy HTx, Mx	Monochromator Filter-based Top or bottom read	30 / 100 μ s n / a	TR-F	Ex: 380 nm \pm 20 nm Em: 650 nm \pm 20 nm
BMG Labtech: PHERASTAR FS / FSX	Filter-based Top or bottom read	40 / 100 μ s n / a	TR-F	Ex 337 nm (HTR-F module) Em 655 nm (HTR-F module)



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Tecan: Infinite M200Pro /Safire / Genios Pro	Monochromator Filter-based Top or bottom read	30 / 100 μ s n / a	TR-F	Ex: 380 \pm 20 nm Em: 650 \pm 20 nm
Perkin Elmer: EnVision / Enspire	Monochromator / Filter-based Top read	40 / 100 μ s n / a	TR-F	Ex: 340 \pm 40 nm (D340) Em: 642 \pm 10 nm (D642)

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:

$$T = \frac{40}{\ln(W1 - W2)}$$

where **T** represents emission lifetime and W1 and W2 represent signals measured at Window 1 and Window 2, respectively. Lifetime values are in μs units, (range ~29 to ~68 μs) and should only be calculated from samples containing MITO-ID[®] Intracellular O₂ Sensor Probe 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 O₂ scale. Lifetime values as calculated above can be converted in oxygen scale using the following default analytical function:

$$O_2\% = 659.3e^{\frac{-T}{8.475}}$$

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 MITO-ID[®] Intracellular O₂ Sensor Probe. 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 Fig. 8.

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:

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

whereby $[O_2]_a$ is oxygen concentration in air-saturated conditions (typically ~20.9%), I_0 and I_a are MITO-ID[®] Intracellular O₂ Sensor Probe 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% CO₂ and 95% humidity, ambient O₂ = ~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 concentrations 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 a higher concentration of MITO-ID[®] Intracellular O₂ Sensor Probe 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 (> 2h) outside a CO₂ incubator as it maintains pH for a prolonged period, without applied CO₂. This is not necessary if using plate reader models equipped with an atmospheric control module where 5% CO₂ can be maintained within the measurement chamber. HEPES based DMEM recipe: Dissolve DMEM powder (Sigma Aldrich D5030, powder) in ~ 900 mL of sterile water. Add 20 mM HEPES, 1 mM Sodium Pyruvate, 20 mM Glucose, 10% FBS, Pen-Strep (if required). Filter sterilize prior to use.

APPENDIX D – TROUBLESHOOTING

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

GENERAL NOTES AND RECOMMENDATIONS

Storage and Stability: On receipt the MITO-ID[®] Intracellular O₂ Sensor Probe reagent should be stored between +2 to +8°C (see Use Before 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. Pre-warm the fluorescence plate reader to measurement temperature and ensure that all culture media and stock solutions to be used in the assay are pre-warmed 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 MITO-ID[®] Intracellular O₂ Sensor Probe 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, MITO-ID[®] Intracellular O₂ Sensor Probe 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 MITO-ID[®] Intracellular O₂ Sensor Probe
6. Contact Instrument Supplier for further options

FREQUENTLY ASKED QUESTIONS:

Q: What do I do if I cannot detect any signal in wells containing cells and MITO-ID[®] Intracellular O₂ Sensor Probe (or I can detect a signal but the slope (rate) appears very low)?

A: Check correct Instrument Settings (Appendix A) - Perform Signal Optimization - Include GOx control (max signal) - Increase cell density. If tested and not resolved, contact techserv-usa@enzolifesciences.com

Q: What do I do if I can detect a signal in wells containing MITO-ID[®] Intracellular O₂ Sensor Probe 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 pre-warmed at 37°C prior to use, reduce plate preparation times.



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NOTE: Some plate readers have inconsistent temperature control. If you suspect this to be the case, consider: – Reduce assay (and equilibration) temperatures to 30°C and avoid outer wells. If tested and not resolved, contact techserv-usa@enzolifesciences.com.

REFERENCES

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3. Dmitriev et al. Anal.Chem. **2012**, 84 (6), 2930-2938



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GLOBAL HEADQUARTERS

Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free: 1.800.942.0430
Phone: 631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA

Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone: +41/0 61 926 89 89
Fax: +41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:

www.enzolifesciences.com