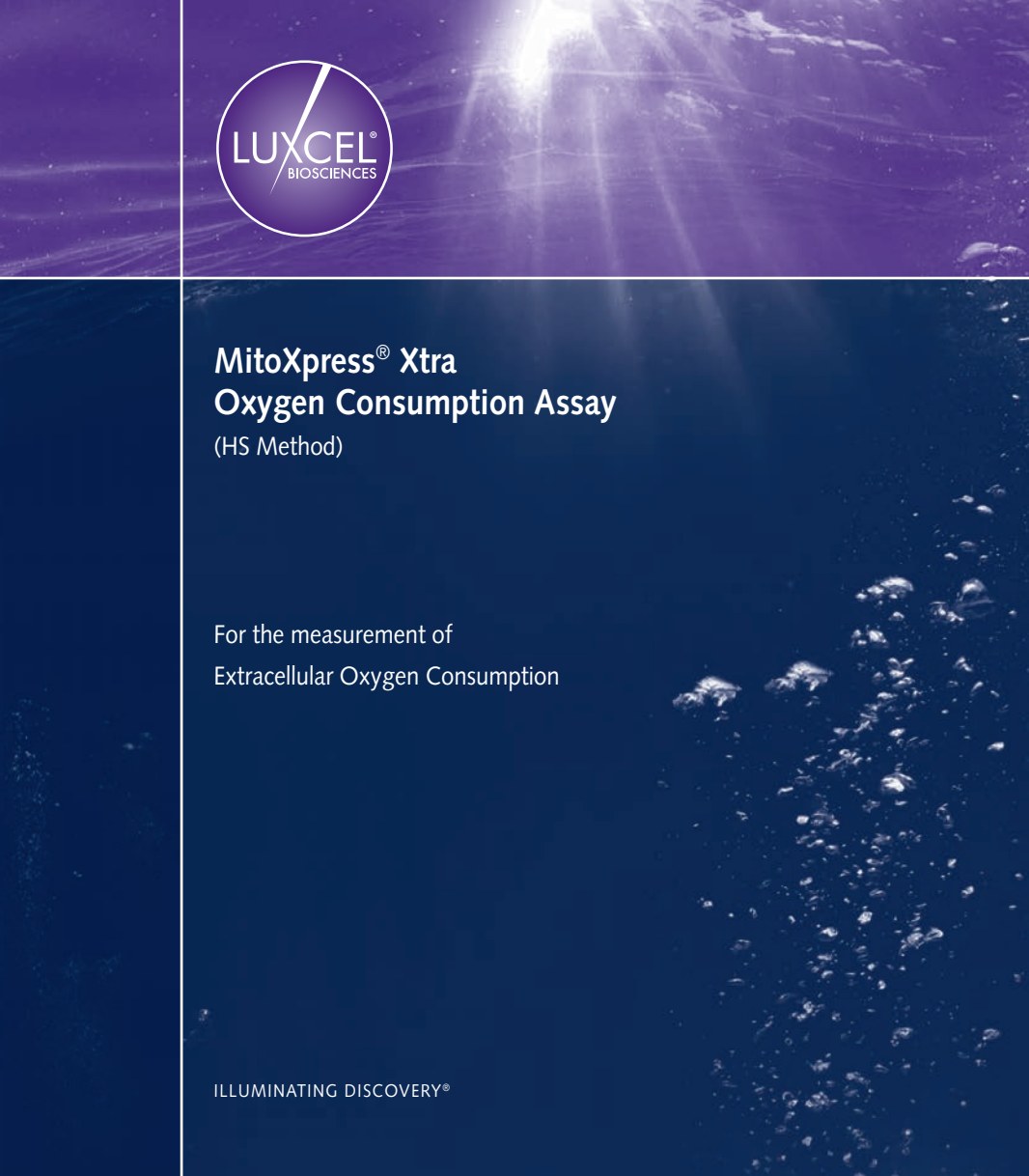


# MitoXpress® Xtra Oxygen Consumption Assay

(HS Method)

For the measurement of

Extracellular Oxygen Consumption

ILLUMINATING DISCOVERY®


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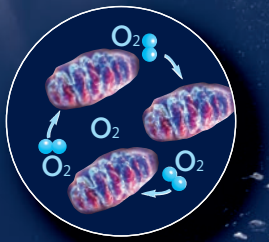
# MitoXpress® Xtra Oxygen Consumption Assay

(HS Method)




For the measurement of Extracellular Oxygen Consumption

For use with:

- Adherent cells;
- Suspension cells;
- Permeabilised cells;
- Isolated mitochondria;
- 3D cultures: tissues, spheroids,
- Raft™ and scaffolds;
- Isolated enzymes;
- Bacteria, yeasts and moulds.

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# General Information

## MATERIALS SUPPLIED

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

| Cat No. | Item | 96 well[1] Quantity / Size | Storage |
|---|---|---|---|
| MX-400 | MitoXpress® Xtra reagent | 1 vial | +4°C |
| HS-100D | HS Mineral Oil | 1 dropper bottle / 15ml | Room Temp / dark |

## STORAGE AND STABILITY

The MitoXpress® Xtra reagent should be stored as follows:

- Dry material between +2 to +8°C (see Use Before date on vial).
- Reconstituted product can be aliquoted at -20°C. Use within one month (avoid freeze thaw).

## ADDITIONAL ITEMS REQUIRED

- Fluorescence plate reader, with suitable filter and plate temperature control.
- 96-well (black wall) clear bottom TC+ plates or standard PS plates for cell culture.

## OPTIONAL ITEMS NOT SUPPLIED

- Repeater pipette (recommended)
- Plate block heater for plate preparation

## SUPPORT

- Visit our website www.luxcel.com.

1. *May also be used in a 384-well format, with one vial of probe sufficient for ~ 200 wells.*

## DESCRIPTION

Luxcel's MitoXpress® Xtra - Oxygen Consumption Assay (HS Method) is a highly flexible 96 or 384-well fluorescence plate reader-based approach, for the direct, real-time analysis of cellular respiration and mitochondrial function. The easy-to-use MitoXpress® Xtra assay allows measurement of extracellular oxygen consumption rates (OCR) with whole cell populations (both adherent and suspension cells), isolated mitochondria, permeabilised cells and a wide range of 3D cultures including: tissues, small organisms, spheroids, scaffolds and matrixes. The assay is also suitable for measurement of isolated enzymes, bacteria, yeasts and moulds.

Scientists at Luxcel Biosciences developed the oxygen-sensing fluorophore known as MitoXpress® Xtra, to overcome the limitations of specialised low throughput instrumentation that were historically used to measure oxygen (eg Clark electrode). The MitoXpress® Xtra reagent is chemically stable and inert, water-soluble and cell impermeable, making it the ideal and scalable mix-and measure reagent for use in a wide range of cell culture conditions - all measured using a fluorescence plate-reader.

In this assay, MitoXpress® Xtra is quenched by $0_2$, through molecular collision, and thus the amount of fluorescence signal is inversely proportional to the amount of extracellular $0_2$ in the sample. Rates of oxygen consumption are calculated from the changes in fluorescence signal over time. The reaction is non-destructive and fully reversible (neither MitoXpress® Xtra nor $O_2$ are consumed), facilitating measurement of time courses and drug treatments.

Luxcel's flexible plate reader format, allows multiparametric or multiplex combination with Luxcel's other products, as well as combination with commonly available reagents to measure glycolysis, LDH, JC-1, MMP ($\Psi$), ROS, and cellular ATP. For example, MitoXpress® Xtra in combination with Luxcel's pH-Xtra® - Glycolysis Assay (Cat No. PH-100) allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states.

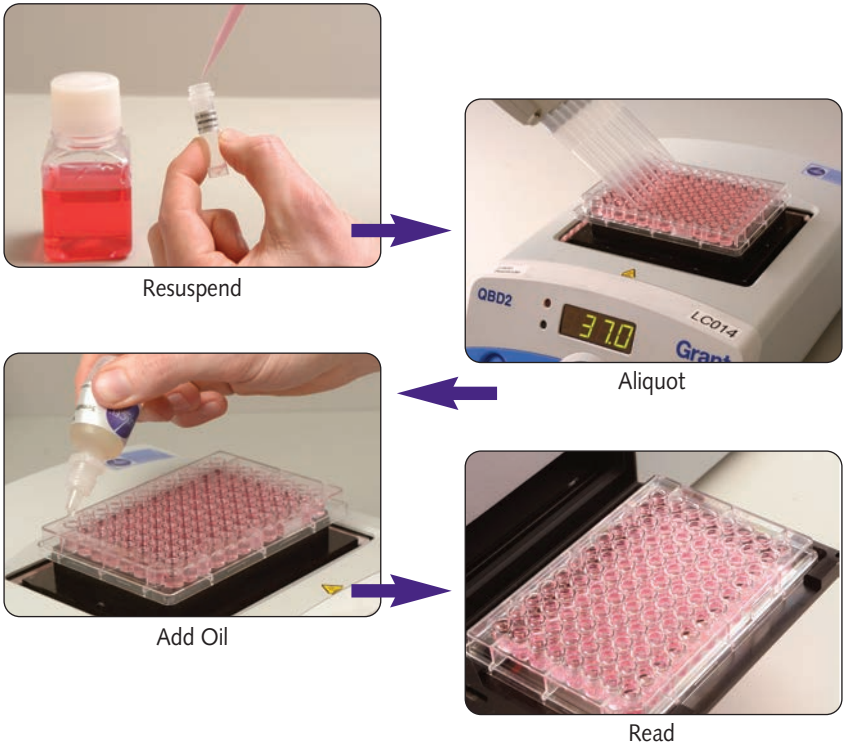


**Figure 1:** Flow diagram showing preparation and use of MitoXpress® Xtra - Oxygen Consumption Assay (HS Method)

## PLATE READER SET-UP

### MEASUREMENT PARAMETERS

MitoXpress® Xtra reagent is a chemically stable and inert, biopolymer-based, cell impermeable oxygen-sensing fluorophore.

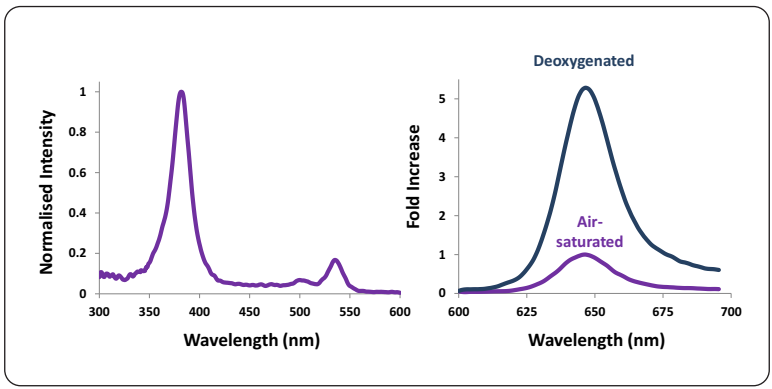


**Figure 2:** Excitation and Emission spectra of MitoXpress® Xtra. Left panel shows normalised excitation (Ex 360-400nm; Peak 380nm). Right panel shows emission (Em 630 - 680nm; Peak 650nm) in oxygenated and deoxygenated conditions.

### INSTRUMENTS AND SETTINGS

Three fluorescence modalities can be successfully used with the MitoXpress® Xtra – Oxygen Consumption Assay (HS Method), depending on plate reader type and instrument setup, as follows:

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

*NOTE: Further details, including instrument, filter selection and measurement settings can be found in Appendix A - Instrument Settings.*

**SIGNAL OPTIMISATION** - *recommended for first time users*

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

**STEP 1:** Reconstitute contents of the MitoXpress® Xtra vial in 1ml of water, PBS or culture media, gently aspirating 3-4 times. *NOTE: Reconstituted probe stock can be stored in the dark between +2 to +8°C for several days or stored as aliquots in water at -20°C for use within one month (avoid freeze thaw).*

**STEP 2:** Prepare 8 replicate wells of a 96-well plate, by adding 150µl pre-warmed culture medium to each well (A1-A4, B1-B4).

**STEP 3:** Add 10µl reconstituted MitoXpress® Xtra reagent to 4 of the replicate wells (A1-A4) and 10µl water, PBS or media to the remaining replicates wells (B1-B4).

**STEP 4:** Promptly add two drops (or 100µl) pre-warmed HS Mineral Oil to all eight replicate wells, taking care to avoid air bubbles. *NOTE: See Appendix B - HS Mineral Oil Pipetting Tips*

**STEP 5:** Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).

**STEP 6:** Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate S:B ratio. *NOTE: For dual read TR-F, calculate S:B for each measurement window.*

For most fluorescence plate readers, set up according to Appendix A - Instrument Settings, MitoXpress® Xtra should return a S:B ≥ 3. Higher readings are expected with TR-F and dual read TR-F measurement. *NOTE: See also Appendix C – Trouble Shooting.*

| | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| **A** | Media + MitoXpress® Xtra + Oil | Media + MitoXpress® Xtra + Oil | Media + MitoXpress® Xtra + Oil | Media + MitoXpress® Xtra + Oil |
| **B** | Media + Oil | Media + Oil | Media + Oil | Media + Oil |

## PERFORMING THE OXYGEN CONSUMPTION ASSAY

### CELL CULTURE AND PLATING

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

- For Adherent cells, seed cells in a 96-well plate at a density (typically 40,000 – 80,000 cells/well) in 200µl culture medium. Incubate overnight in a $CO_2$ incubator at 37°C.
- For Suspension cells, seed on the day of assay in 150µl culture medium at a density of ~ 4 x $10^6$/ml.

Visit our website www.luxcel.com for more information on the use of MitoXpress® Xtra with permeabilised cells, 3D cultures, tissues, spheroids, Raft™ and scaffolds, isolated enzymes, bacteria, yeasts and moulds.

### PRE-ASSAY PREPARATION

- Reconstitute the contents of the MitoXpress® Xtra vial in 1ml of water, PBS or culture media, gently aspirating 3-4 times (Figure 3). *NOTE: Reconstituted probe stock can be stored in the dark between +2 to +8°C for several days or stored as aliquots in water at -20°C for use within one month (avoid freeze thaw).*

- Prepare test compounds, controls and dilutions as desired. Typical controls are Antimycin A (Complex III inhibitor), FCCP (ETC uncoupler) and Glucose Oxidase (GOx; positive signal control).

*NOTE: We recommend that all culture media and stock solutions to be used in the assay are pre-warmed at 37°C prior to use. Use a plate block heater for plate preparation and pre-warm the fluorescence plate reader to measurement temperature.*

**Figure3:** Reconstitution of MitoXpress® Xtra vial

## TYPICAL ASSAY

To assess Oxygen Consumption or to investigate the effect of a compound on electron transport chain function (ETC; oxidative phosphorylation), cells are treated immediately prior to measurement. *NOTE: We recommend the use of triplicate wells for each treatment.*

**STEP 1:** Remove spent culture medium from all assay wells and replace with 150µl of fresh culture media (Figure 4). *NOTE: We recommend always leaving two wells (H11 and H12) free from the addition of MitoXpress® Xtra reagent, for use as Blank Controls.* Add 150µl of fresh culture media to these Blank Control wells also.

**STEP 2**: Add 10µl reconstituted MitoXpress® reagent to each well, except those wells for use as Blank Controls. Add 10µl of fresh culture media to these Blank Control wells. *NOTE: If plating a full 96-well plate of assays, we recommend combining Step 1 and Step 2 by adding the 1ml of reconstituted MitoXpress® Xtra reagent to 15ml pre-warmed fresh culture media and using a multi-channel pipette to add 150µl of MitoXpress® Xtra in media stock to each well (Figure 4).* Add 150µl of fresh culture media only (no MitoXpress® Xtra) to the Blank Control wells.

**STEP 3:** Test compound stock or vehicle (typically 1-10µl) may be added at this point if desired. *NOTE: We recommend keeping the volume of added compound low to minimise any potential effects of solvent vehicle.*


**Figure 4:** Aliquoting fresh media (+/- MitoXpress® Xtra)

**STEP 4:** Promptly seal each well by adding two drops (or 100µl) pre-warmed HS Mineral Oil, taking care to avoid air bubbles (Figure 5).
*NOTE: Small variations in the volume of oil (between 90-110µl) should not adversely affect the readings using MitoXpress® Xtra.*
*See also Appendix B - HS Mineral Oil Pipetting Tips.*

**STEP 5:** Read the plate immediately in a fluorescence plate reader, with the set-up as described in Appendix A - Instrument Settings (Figure 6). The plate should be measured kinetically for >90 minutes. When measurement is completed, remove the plate and save measured data to file.


**Figure 5:** Adding pre-warmed HS Mineral Oil

**Optional Controls:**

- **Signal Controls:** Leave 2 or 3 wells free from the addition of cells for use as Signal Controls. Add 150µl of fresh culture media +10µl of reconstituted MitoXpress® Xtra reagent to each well.

- **Positive Controls:** Leave 2 or 3 wells free from the addition of cells for use as Positive Controls. Add 150µl of fresh culture media + 10µl of (1mg/ml) Glucose Oxidase stock solution (in water) + 10µl reconstituted MitoXpress® Xtra reagent to each well.

- **Negative Controls:** To 2 or 3 wells containing cells, add 1µl of (150 µM) Antimycin A stock solution (in DMSO) + 10µl reconstituted MitoXpress® Xtra reagent.

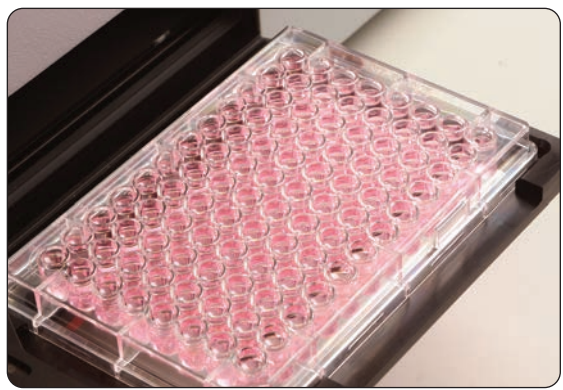

**Figure 6:** Reading the assay plate

## ANALYSIS

*NOTE: We recommend that all first time users perform a Signal Optimisation test, as described. Signal and Blank Control wells may also be included.*

### ASSESSING OXYGEN CONSUMPTION

Plot the Blank Control well-corrected MitoXpress® Xtra Intensity or Lifetime values versus Time (mins; Figure 7). Select the linear portion of the signal profile (avoiding any initial lag or subsequent plateau) and apply linear regression to determine the slope (OCR) and correlation coefficient for each well. *NOTE: This approach is preferable to calculating a slope from averaged profiles.*

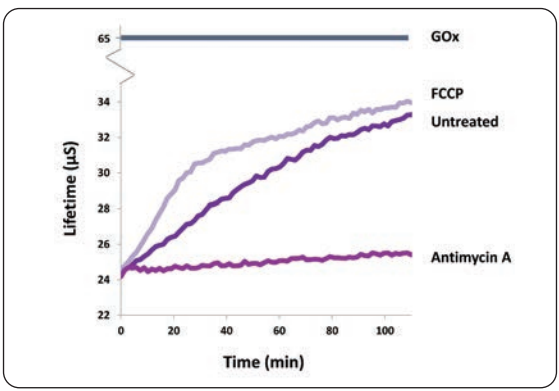


**Figure 7:** Typical Lifetime profile of MitoXpress® Xtra for adherent cells, treated with different ETC compounds, including Antimycin A (recommended as a Negative Control). The effect of Glucose Oxidase as a positive Signal Control is illustrated schematically. *NOTE: If using FCCP it is strongly recommended to perform a dose titration, since FCCP exhibits a bell-shaped response.*

Tabulate the slope values for each test sample, calculating appropriate average and standard deviation values across replicate wells. If optional Signal Control wells are included, the slope obtained for the Signal Control (sample without cells) should be subtracted from all test values.

Data analysis templates are available from some plate reader manufacturers, specifically configured to automate the analysis of Luxcel's MitoXpress® range of assays. Microsoft Excel templates are also available through our website www.luxcel.com.

## PLOTTING A DOSE RESPONSE CURVE

To generate a dose response curve, plot the data generated as outlined above against the corresponding compound concentration (Figure 8).

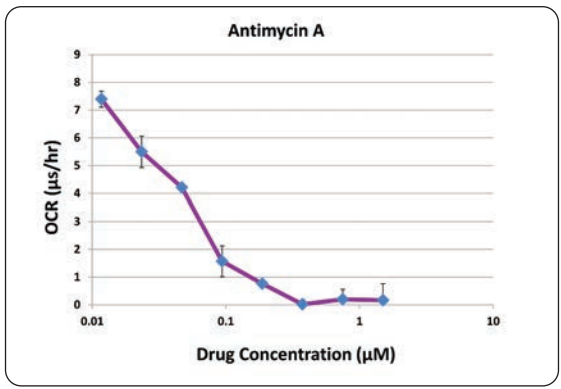


**Figure 8:** The dose response curve presented here is an example of the data typically produced with this assay. Drug concentration (µM) versus calculated slope (µs/hour) demonstrates that this drug causes inhibitory response on cellular respiration.

## CELLULAR ENERGY FLUX ANALYSIS

Multiparametric (or multiplex) combination of MitoXpress® Xtra - Oxygen Consumption Assay (HS Method) together with Luxcel's pH-Xtra® - Glycolysis Assay (Cat No. PH-100) allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states (Figure 9).

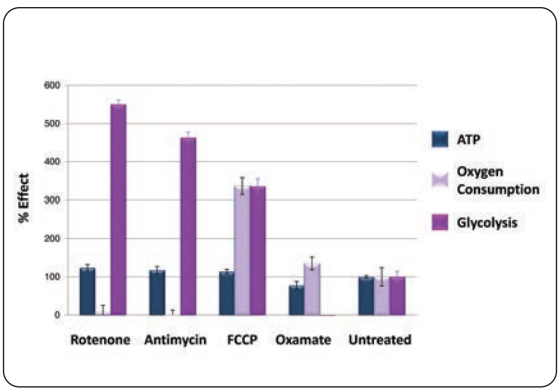


**Figure 9:** Cellular Energy Flux for HepG2 cells, treated with a combination of drug compounds modulating the ETC or inhibiting lactate production, shown as a percentage relative to untreated control cells. Comparative measurements with MitoXpress® Xtra and pH-Xtra®, show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP; measured 1h post-treatment using Promega Cell Titer-Glo®).

## APPENDIX A - INSTRUMENT SETTINGS

Three fluorescence modalities can be successfully used depending on plate reader type and instrument setup. *NOTE: We strongly recommend only using fluorescence plate readers equipped with temperature control.*

**Basic: Intensity Measurement**
Measurement of signal Intensity (sometimes referred to as Prompt) provides flexibility to use a very wide range of commonly available fluorescence, monochromator or filter-based plate readers. Optimal wavelengths are 380nm excitation and 650nm for emission, with detection Gain parameters (PMT) typically set at medium or high. *NOTE: MitoXpress® Xtra should return a S:B ≥ 3*

**Standard: TR-F Measurement**
Increased levels of performance can be achieved by using time-resolved fluorescence (TR-F). TR-F measurement reduces non-specific background and increases probe sensitivity. Optimal delay time is ~30µs and gate (integration) time is 100µs. *NOTE: MitoXpress® Xtra should return a S:B > 3 S:B ~10 are typical.*

**Advanced: Dual-Read TR-F (Lifetime)**
Optimal performance can be achieved using dual-read TR-F in combination with subsequent ratiometric Lifetime calculation, to maximise dynamic range (Figure 10). *NOTE: MitoXpress® Xtra should return a S:B ≥ 3 and S:B up to 60 are possible.*

Optimal dual-delay and gate (integration) times:
- Integration window 1: 30µs delay ($D_1$), 30µs measurement time ($W_1$)
- Integration window 2: 70µs delay ($D_2$), 30µs measurement time ($W_2$)

## DUAL-READ TR-F AND LIFETIME ILLUSTRATED

Dual-read TR-F and subsequent Lifetime calculation allows measurement of the rate of fluorescence decay of the MitoXpress® Xtra reagent, and can provide measurements of oxygen consumption that are more stable and with a wider dynamic range than measuring signal Intensity. *NOTE: S:B for Integration window 2 is recommended to be ≥ 10 to allow accurate Lifetime calculation.*

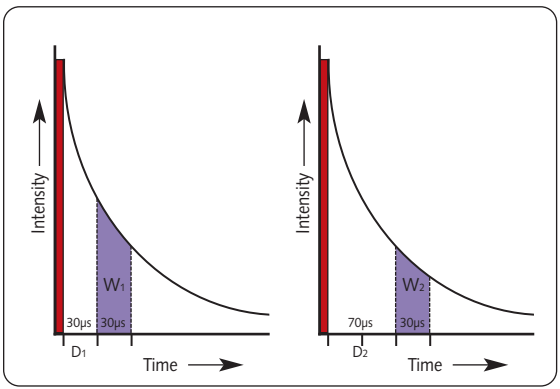


**Figure 10:** Illustrating dual-read TR-F measurement.

Use the dual intensity readings to calculate the corresponding Lifetime (µs) using the following transformation:

$$Lifetime\ (\mu s)[\tau] = (D_2 - D_1)/\ln(W_1/W_2)$$

Where $W_1$ and $W_2$ represent the two (dual) measurement windows and $D_1$ and $D_2$ represent the delay time prior to measurement of $W_1$ and $W_2$ respectively. This provides Lifetime values in microsecond units (µs) at each measured time point for each individual sample (Figure 10). *NOTE: Lifetime values should be in the range ~22 to ~68µs, and should only be calculated from samples containing MitoXpress® Xtra reagent. Lifetime values should not be calculated from blank wells.*

## RECOMMENDED INSTRUMENT AND MEASUREMENT SETTINGS

| Instrument | Optical Configuration | Integration 1 ($D_1$ / $W_1$) Integration 2 ($D_2$ / $W_2$) | Mode | Ex (nm) Em (nm) |
|---|---|---|---|---|
| **BMG Labtech:*** FLUOStar Omega / POLARstar Omega (CLARIOstar)** | Filter-based Top or bottom read | 30 / 30µs 70 / 30µs | Dual-read TR-F (Lifetime)*** | Ex 340 ± 50nm (TR-EX L) Em 650 ± 50nm (BP-655) |
| **BMG Labtech:*** PHERAstar FS | Filter-based Top read | 40 / 100µs n/a | TR-F | Ex 337nm (HTRF Module) Em 665nm (HTRF Module) |
| **BMG Labtech:*** FLUOStar Optima / POLARstar Optima | Filter-based Top or bottom read | 30 / 100µs n/a | TR-F | Ex 340 ± 50nm (TR-EX L) Em 655 ± 50nm (BP-655) |
| **Perkin Elmer:** VICTOR series / X4, X5 | Filter-based Top read | 30 / 30µs 70 / 30µs | Dual read TR-F (Lifetime) | Ex 340 ± 40nm (D340) Em 642 ±10nm (D642) |
| **Perkin Elmer:** EnVision, EnSpire | Filter-based Top read | 40 / 100µs n/a | TR-F | Ex 340nm ± 60nm (X340) Em 650nm ± 8nm (M650) |
| **BioTek:*** Synergy H1, H4, HT, 2 (Cytation 3)** | Filter-based Top or bottom read | 30 / 30µs 70 / 30µs | Dual read TR-F (Lifetime) | Ex 380 ±20nm Em 645 ± 15nm |
| **BioTek:** Mx, H1m | Monochromator / Filter-based Top or bottom read | 30 / 100µs n/a | TR-F | Ex 380 ± 20nm Em 650±15nm |
| **Tecan:** Infinite / Safire / Genios Pro | Monochromator / Filter-based Top or bottom read | 30 / 100µs n/a | TR-F | Ex 380 ± 20nm Em 650 ± 20nm |
| **Mol. Devices:** SpectraMax / Flexstation / Gemini | Monochromator-based Top or bottom read | n/a n/a | Intensity (Prompt) | Ex 380nm Em 650nm |
| **Hidek:** SENSE / CHAMELEON | Filter-based Top or bottom read | 30 / 100µs n/a | TR-F | Ex 390 ± 20nm Em 660 ± 10nm |
| **Thermo:** Varioskan / Fluoroscan Ascent | Monochromator / Filter-based Top or bottom read | 30 / 100µs n/a | TR-F | Ex 380nm Em 650nm |

**Notes:** * Assay-specific protocols and notes are available from manufacturer for MitoXpress® Xtra.
** Assay-specific protocols in development (contact TechSupport@Luxcel.com)
*** TR-F head must be installed

## APPENDIX B - HS MINERIAL OIL PIPETTING TIPS

HS Mineral Oil is provided in an easy to use dropper bottle for convenience, although we recommend a repeater pipette for routine use.


**Figure 4:** Add Oil

- **Dropper Bottle:** Invert the pre-warmed dropper bottle and apply gentle pressure, just sufficient to prime the oil in the bottle tip. Apply two (2) drops to each well, touching each drop as it is formed to the side of the well to allow it to run down onto the surface of the culture media.

- **Repeater Pipette:** Use of a repeater pipette saves time and helps to maintain more precise incubation times. Prepare the repeater syringe tip by trimming ~ 3-4mm off the tip at a 45° angle. Remove the internal nozzle cap from the dropper bottle and slowly pick up the pre-warmed HS Mineral Oil (avoid pipetting up and down, as this can cause bubbles) and dispense 100µl to each well at an angle of ~45°, allowing the oil to flow the side of each well. *NOTE: Small variations in the volume of HS Minerial Oil (between 90-110µl) should not adversely effect the readings using MitoXpress® Xtra.*

## APPENDIX C – TROUBLE SHOOTING

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

### GENERAL NOTES AND RECOMMENDATIONS

**Storage and Stability:** On receipt the MitoXpress® Xtra reagent should be stored between +2 to +8°C (see Use Before date on vial). Reconstituted probe stock can be stored in the dark between +2 to +8°C for several days or stored as aliquots in water at -20°C for use within one month (avoid freeze thaw).

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

**Plates:** We recommend 96 or 384-well black wall / clear bottom TC+ plates, although standard clear wall PS plates for cell culture may also be used.

**Temperature:** We recommend the use of a plate block heater for plate preparation, 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 Optimisation and Use of Controls:** We recommend performing a signal optimisation check, especially for first time users, and inclusion of blank and optional additional control wells as described.

**Pipetting HS Oil:** Take care when dispensing the HS Mineral Oil to avoid bubbles. Apply HS Mineral Oil allowing it to run down the inside surface of each well. Do not shake or rapidly aspirate the HS Mineral Oil.

**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. Work rapidly once the MitoXpress® Xtra reagent has been added, to reduce the potential for assay variability.

**Cell Type and Cell Density:** Since the MitoXpress® Xtra reagent measures extracellular Oxygen Consumption, the amount of signal change will be directly dependent on the rate of cellular respiration of the cell type being measured. We recommend using as high a cell density per well as practical as a starting point, and reducing cell numbers as required. Not all cell types may consume sufficient oxygen for detection.

## SIGNAL TO BLANK (S:B) OPTIMISATION

For most fluorescence plate readers, set up according to Appendix A - Instrument Settings, MitoXpress® Xtra should return a signal to blank ratio ≥ 3. Higher readings are expected with TR-F and dual read TR-F measurement. 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 volume of MitoXpress® Xtra (15µl).
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 MitoXpress® Xtra (or I can detect a signal but the slope (rate) appears very low)?
A: Check correct Instrument Settings (Appendix A) - Perform Signal Optimisation - Include GOx control (max signal) - Increase cell density. If tested and not resolved, contact TechSupport@luxcel.com

Q: What do I do if I can detect a signal in wells containing cells and MitoXpress® Xtra, but the slope (rate) 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.

*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 TechSupport@luxcel.com.*

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## RELATED PRODUCTS

- pH-Xtra® – Glycolysis Assay (Cat No. PH-100)
- MitoXpress® Intra – Intracellular $O_2$ Assay (Cat No. MX-300)
- GreenLight® 960 – Microbial Detection Assay (Cat No. GL-960)


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