Mito-ID® $O_2$ Extracellular Sensor Kits

ENZ-51044-K100/500  Mito-ID® Extracellular $O_2$ Sensor Kit
ENZ-51045-K100/500  Mito-ID® Extracellular $O_2$ Sensor Kit (High Sensitivity)
ENZ-51047    Mito-ID® Extracellular $O_2$ Sensor Probe
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INTRODUCTION

Oxygen consumption is one of the most informative and direct measures of mitochondrial function. Mitochondria have been shown to play a central role in cellular metabolism, bioenergetics, and apoptosis. Mitochondrial dysfunction is implicated in numerous disease states, including cancer, obesity, neurodegeneration and ischemia. A major mechanism of drug-induced toxicity has been linked to mitochondria dysfunction. Limitations with the traditional methods of measuring oxygen consumption, includes low throughput and high complexity. The Mito-ID® O₂ Extracellular Sensor kit resolves these limitations by providing a direct, real-time measurement of extracellular oxygen consumption rate (OCR) on a fluorescence plate reader.

The Mito-ID® Extracellular O₂ Sensor Kit (ENZ-51044) and Mito-ID® Extracellular O₂ Sensor Kit (High Sensitivity) (ENZ-51045) provides a highly flexible, 96- or 384-well, fluorescence plate reader based approach, for the direct, real time analysis of cellular respiration and mitochondrial function.

This easy-to-use assay allows measurement of extracellular oxygen consumption rates (OCR) with whole cell populations (both adherent and suspension cell), isolated mitochondria, permeabilized cells and a wide range of 3D cultures including: tissues, small organisms, spheroids, scaffolds and matrices. The assay is also suitable for measurement of isolated enzymes, bacteria, yeasts and molds.

Drugs/compounds of interest can be evaluated for their dose-dependent toxicity effects assessing the impact of a given manipulation on cellular function. These results can help in applications such as mode of drug action elucidation, screening for antimicrobial compounds, the assessment of bacterial load and the optimization of culture conditions.

The flexible plate reader format allows multiparametric or multiplex combination with Enzo Life Sciences other products such as Mito-ID® Extracellular pH Sensor Probe (ENZ-51048), JC-10, ATP. For example, O₂ Sensor Probe in combination with pH Sensor Probe allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis, and the analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states.
APPLICATIONS

- Determine mitochondria dysfunction
  (cell based, including whole cells, tissue, enzymes and small organisms)
- Compound Screening and toxicity effects.
  Determine IC_{50} values
- Cellular and Microbial metabolism studies
- Multiplexing (e.g. ETC and glycolytic flux analysis using Mito-ID® pH Extracellular Sensor Probe (ENZ-51048).)

**NOTE:** The Mito-ID® Extracellular O_2 Sensor Kit (ENZ-51044) is recommended for applications using isolated mitochondria, bacteria and select tissues.

**NOTE:** The Mito-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045) is recommended for cell-based applications

ASSAY PRINCIPLE

The 96/384-well plate assay is based on the ability of oxygen to quench the excited state of the water-soluble Mito-ID® Extracellular O_2 Sensor Probe. As the test material respires (e.g., isolated mitochondria, cell populations, small organism, tissues and enzymes), O_2 is depleted in the surrounding solution/environment, which is seen as an increase in probe fluorescence signal. Changes in oxygen consumption, which reflect changes in mitochondrial activity, can be correlated with the signal strength of the Mito-ID® Extracellular O_2 Sensor Probe over time. The assay is non-chemical and reversible.

Workflow diagram showing preparation and use of ENZ-51045 Mito-ID® Extracellular O_2 Sensor Kit (High Sensitivity).
KIT CONTENTS

<table>
<thead>
<tr>
<th>Kit #</th>
<th>Reagent Description</th>
<th>Volume and Quantity</th>
<th>Reactions</th>
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</thead>
<tbody>
<tr>
<td>ENZ-51044-K100</td>
<td>• Mito-ID® Extracellular O₂ Sensor Probe</td>
<td>1 vial 5 vials</td>
<td>100</td>
</tr>
<tr>
<td>ENZ-51044-K500</td>
<td>• Mito-ID® Oil</td>
<td>1 bottle, 12ml 1 bottle, 60ml</td>
<td>500</td>
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<tr>
<td><strong>The Mito-ID® Extracellular O₂ Sensor Kit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENZ-51045-K100</td>
<td>• Mito-ID® Extracellular O₂ Sensor Probe</td>
<td>1 vial 5 vials</td>
<td>100</td>
</tr>
<tr>
<td>ENZ-51045-K500</td>
<td>• Mito-ID® HS Oil</td>
<td>1 dropper bottle, 15ml 5 dropper bottles, 15ml each</td>
<td>500</td>
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<tr>
<td><strong>The Mito-ID® Extracellular O₂ Sensor Kit (High Sensitivity)</strong></td>
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<tr>
<td>ENZ-51047*</td>
<td>• Mito-ID® Extracellular O₂ Sensor Probe</td>
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</tbody>
</table>

* Results can vary. A comparable mineral oil is required to perform application assays.

STORAGE

Store dry material between +2 to +8°C away from light. Reconstituted product can be aliquoted in water and stored at -20°C. Use within one month (avoid freeze/thaw).

STABILITY

12 months upon receipt

FORMAT

Easy “mix and measure” plate-based assay using standard 96- or 384-well plates.
REQUIRED AND OPTIONAL MATERIALS

1. Repeater pipette (for oil addition) and multiple-channel pipette
2. Plate heater
3. 96-well (black wall) clear bottom TC+ plates or standard clear polystyrene plates for cell culture.
4. Cells or isolated mitochondria
5. Cell culture medium (for cells)
6. Measurement buffer (for isolated mitochondria)
7. Compounds of interest
8. Fluorescence plate reader
9. Mitochondrial substrate (succinate, glutamate or malate) (for isolated mitochondria assay)
10. ADP (for isolated mitochondria assay)

PRECAUTIONS

1. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately.
2. Interpretation of the results is the sole responsibility of the user.

LIMITATIONS

- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding
TECHNICAL HINTS

- Avoid foaming or bubbles when mixing, reconstituting or adding components (especially the oil).
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- The buffers, plate and oils prepared should be equilibrated to assay temperature (block heater), before measurement is started.
- Complete removal of all solutions and buffers during wash steps.
- For application of High Sensitivity mineral oil – if using a repeater pipette, for greater speed, trim 3-4mm off the tip at a 45° angle.
- Refer to the guide in the Instrument and Measurement Settings section, for recommended settings for your plate reader.
- Perform a Signal Optimization step (especially first time users).
- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
PLATE READER SET-UP

MEASUREMENT PARAMETERS

The Mito-ID® Extracellular O₂ Sensor reagent is a chemically stable and inert, biopolymer-based, cell impermeable oxygen-sensing fluorophore.

![Excitation and Emission spectra of Mito-ID® Extracellular O₂ Sensor reagent.](image)

Excitation and Emission spectra of Mito-ID® Extracellular O₂ Sensor reagent. Left panel shows normalized excitation (Ex 360-400nm; Peak 380nm). Right panel shows emission (Em 630 - 680nm; Peak 650nm) in oxygenated and deoxygenated conditions.

INSTRUMENT SETTINGS

Three fluorescence modalities can be successfully used with the Mito-ID® Extracellular O₂ Sensor Kit, Two depending on plate reader type and instrument setup, as follows:

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

**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 for excitation and 650nm for emission, with detection Gain parameters (PMT) typically set at medium or high. **NOTE**: Signal Optimization test may 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 sensitivity. Optimal delay time is ~30µs and gate (integration) time is 100µs. **NOTE**: Signal Optimization test may return a S:B ~10.
Advanced: Dual-Read TR-F (Lifetime)

Optimal performance can be achieved using dual-read TR-F in combination with subsequent ratiometric lifetime calculation, to maximize dynamic range. **NOTE:** Signal Optimization test may return a S:B up to 60.

Optimal dual-delay and gate (integration) times:

- Integration window 1: 30µs delay (D1), 30µs measurement time (W1)
- Integration window 2: 70µs delay (D2), 30µs measurement time (W2)

**DUAL-READ TR-F**

Dual-read TR-F and subsequent lifetime calculation allows measurement of the rate of fluorescence decay of the Mito-ID® Extracellular O₂ Sensor Kit, 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.

**Illustrating dual-read TR-F Measurement**

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

\[
\text{Lifetime (µs)}[\tau] = \frac{(D_2-D_1)}{\ln(W_1/W_2)}
\]

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 Mito-ID® Extracellular O₂ Sensor Probe. Lifetime values should not be calculated from blank wells.
SIGNAL OPTIMIZATION

NOTE: Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C).

STEP 1: Reconstitute contents of the Extracellular O₂ Consumption Reagent in 1ml of water, PBS or culture media, gently aspirating 3-4 times.

STEP 2: Prepare eight 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 Extracellular O₂ Consumption 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 High sensitivity mineral oil to all eight replicate wells, taking care to avoid air bubbles. NOTE: Use repeater pipette for most accurate and speedy addition.

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 Signal to Blank (S:B) ratio. NOTE: For dual read TR-F, calculate S:B for each measurement window.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Media + O₂ Reagent + Oil</td>
<td>Media + O₂ Reagent + Oil</td>
<td>Media + O₂ Reagent + Oil</td>
<td>Media + O₂ Reagent + Oil</td>
</tr>
<tr>
<td>B</td>
<td>Media + Oil</td>
<td>Media + Oil</td>
<td>Media + Oil</td>
<td>Media + Oil</td>
</tr>
</tbody>
</table>
METHODS AND PROCEDURES
The procedures described in this manual assume that the user is familiar with the basic principles and practices of fluorescence microscopy/plate reader. Use sterile tissue conditions.

The application protocols included might require optimization and should be solely used as a guideline.

NOTE: PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Before starting the procedure, allow provided kit reagents to warm to room temperature for about 5-10 minutes. Upon heating to room temperature, briefly centrifuge the Mito-ID® Extracellular O₂ Sensor Probe vial to gather the contents at the bottom of the tube. Typical assay will be performed at 37°C. Prepare and use appropriate controls for each experiment.

CELL CULTURE AND PLATING
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 CO₂ incubator at 37°C.

TIP: Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

For Suspension cells, seed on the day of assay in 150µl culture medium at a density of ~ 4 x 10⁶/ml.

For Isolated Mitochondria, dilute to the desired concentration in measurement buffer and add 150µl to each test well (typically in the range of 0.125-1.5mg/ml final concentration, depending on the substrate(s) used and whether measuring Basal [state 2] or ADP-stimulated respiration rate [state 3])

NOTE: Always leave two wells (H11 and H12) free from the addition of Mito-ID® Extracellular O₂ Sensor Probe, as Blank Controls.
REAGENT PREPARATION

Reconstitute the contents of the *Mito-ID*® *Extracellular* O₂ *Sensor Probe* in 1ml of water, PBS, culture media or buffer, gently aspirating 3-4 times.

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

For use with isolated mitochondria, prepare measurement buffer (pH 7.4) according to the following table:

<table>
<thead>
<tr>
<th>CONSTITUENT</th>
<th>CONCENTRATION (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>250</td>
</tr>
<tr>
<td>KCl</td>
<td>15</td>
</tr>
<tr>
<td>EGTA</td>
<td>1</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>30</td>
</tr>
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</table>

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

ISOLATED MITOCHONDRIA ASSAY OPTIMIZATIONS

Prepare a six point dilution series of mitochondrial preparation in respiration buffer in 1.5ml total volume for each concentration. Starting mitochondrial concentrations recommended for different substrates and respiration states are:

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate / malate</td>
<td>1.5mg ml⁻¹</td>
</tr>
<tr>
<td>Glutamate / malate / ADP</td>
<td>1.0mg ml⁻¹</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.0mg ml⁻¹</td>
</tr>
<tr>
<td>Succinate / ADP</td>
<td>0.5mg ml⁻¹</td>
</tr>
</tbody>
</table>

Typical final concentrations:

Final substrate concentration of 25mM (succinate) or 12.5/12.5mM (glutamate/malate)

Final ADP concentration: 1.65mM
ASSAY PROCEDURE

To assess oxygen consumption or to investigate the effect 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: For adherent cells: remove spent culture medium from all assay wells and replace with 150µl of fresh culture media. For Isolated Mitochondria: dissolve substrate (succinate or glutamate/malate) and ADP in measurement buffer and add 50µl to test wells already containing 150µl of mitochondria in Buffer, giving a final substrate concentration of 25mM (succinate) or 12.5/12.5mM (glutamate/malate) and a final ADP concentration of 1.65mM.

NOTE: We recommend always leaving two wells (H11 and H12) free from the addition of Extracellular O₂ Consumption Probe, for use as Blank Controls. Add 150µl fresh culture media (cell-based assay) or 200µl measurement buffer (isolated mitochondria assay) to these Blank Control wells.

STEP 2: Add 10µl reconstituted Mito-ID® Extracellular O₂ Sensor Probe 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 2 by adding the 1ml of reconstituted O₂ Sensor Probe to 15ml pre-warmed fresh culture media, and using a multi-channel pipette to add 150µl Mito-ID® Extracellular O₂ Sensor Probe in media stock to each well. Add 150µl fresh culture media (cell-based assay) or 200µl measurement buffer (isolated mitochondria assay) to these 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 minimize any potential effects of solvent vehicle.

STEP 4: Promptly seal each well by adding two drops (or 100µl) pre-warmed High Sensitivity mineral oil, taking care to avoid air bubbles. NOTE: Plate preparation time should be kept to a minimum. Use repeater pipette for most accurate and speedy addition.

STEP 5: Read the plate immediately in a fluorescence plate reader. The plate should be measured kinetically for >90 minutes. When measurement is completed, remove the plate and save measured data to file.
For Isolated Mitochondria: measure Mito-ID® Extracellular O₂ Sensor Kit at 1.5 min intervals for 10–30 minutes.

**NOTE:** Mitochondria are freshly prepared as per user’s protocol and should not be left on ice longer than recommended in the literature. Measurement buffers should be prepared freshly on the day of measurement.

**ANALYSIS**

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

**ASSESSING OXYGEN CONSUMPTION**

Plot the Blank Control well-corrected Mito-ID® Extracellular O₂ Sensor Kit intensity or lifetime values versus time (mins). 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.

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.

Typical lifetime profile of Mito-ID® Extracellular O₂ Sensor Kit 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.
PLOTTING A DOSE RESPONSE CURVE

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

The dose response curve presented here is an example of the data typically produced with this assay. Drug Concentration (µM) versus O₂ Consumption calculated slope (µs/hour) demonstrates that this drug causes inhibitory response on cellular respiration.
## INSTRUMENT AND MEASUREMENT SETTINGS

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Optical Configuration</th>
<th>Integratio n 1 (D1 / W1)</th>
<th>Integratio n 2 (D2 / W2)</th>
<th>Mode</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
</tr>
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<tbody>
<tr>
<td>BioTek: Cytation 3 / 5</td>
<td>Filter-based Top or bottom read</td>
<td>30 / 30µs 70 / 30µs</td>
<td></td>
<td>Dual read TR-F (Lifetime)</td>
<td>Ex 380 ± 20nm</td>
<td>Em 645 ± 15nm</td>
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<td>TR-F</td>
<td>Ex 380 ± 20nm</td>
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<td>Filter-based Bottom read</td>
<td>30 / 30µs 70 / 30µs</td>
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<td>Dual-read TR-F (Lifetime)</td>
<td>Ex 340 ± 50nm (TR-EX) Em 665 ± 50nm or Em 645 ± 10nm with LP-TR Dichroic</td>
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<td>Ex 340 ± 50nm (TR-EXL) Em 655 ± 50nm (BP-655)</td>
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<td>Ex 337nm (HTRF Module) Em 665nm (HTRF Module)</td>
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<td>intensity (Prompt)</td>
<td>Ex 380nm</td>
<td>Em 650nm</td>
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<td>40 / 100µs n/a</td>
<td></td>
<td>TR-F</td>
<td>Ex 380nm ± 20nm</td>
<td>Em 650nm ± 20nm</td>
</tr>
<tr>
<td>Perkin Elmer: EnVision</td>
<td>Filter-based Top read</td>
<td>40 / 100µs n/a</td>
<td></td>
<td>TR-F</td>
<td>Ex 340nm ± 60nm (X340) Em 650nm ± 8nm (M650)</td>
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<td>Perkin Elmer: VICTOR series / X4 / X5</td>
<td>Filter-based Top read</td>
<td>30 / 30µs 70 / 30µs</td>
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<td>Dual read TR-F (Lifetime)</td>
<td>Ex 340 ± 40nm (D340) Em 642 ±10nm (D642)</td>
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<tr>
<td>Tecan: Infinite M1000Pro / F200Pro</td>
<td>Monochromator / Filter-based Top or bottom read</td>
<td>30 / 30µs 70 / 30µs</td>
<td></td>
<td>Dual read TR-F (Lifetime)</td>
<td>Ex 380 ± 20nm</td>
<td>Em 650 ± 20nm</td>
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<tr>
<td>Tecan: Infinite M200Pro / Saffire / GeniosPro</td>
<td>Monochromator / Filter-based Top or bottom read</td>
<td>30 / 100µs n/a</td>
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<td>TR-F</td>
<td>Ex 380 ± 20nm</td>
<td>Em 650 ± 20nm</td>
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COMPLETE CELL-BASED SCREENING FOR DRUG INDUCED MITOCHONDRIAL DYSFUNCTION

NOTE: This protocol should be used with ENZ-51045 which includes the Mito-ID® High Sensitivity (HS) Oil and Mito-ID® Extracellular pH Sensor Probe (ENZ-51048). The Mito-ID® HS Oil should be pre-warmed to 37°C. All other reagents should be warmed to 37°C prior to assay and freshly prepared on the day of the experiment. Procedure outlined below is based on using a 384-well plate format.

Overview of Setup

The Mito-ID® Extracellular O₂ Sensor Probe and Mito-ID® pH Extracellular Probe can be multiplexed or used in parallel (as shown). This will allow for simultaneous glycolytic flux and ETC analysis. Compound / drug of interest should be serially diluted.

1. Grow an appropriate cell line of choice in a compatible medium. Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for an experiment.

   NOTE: Consideration should be taken when choosing a cell line to allow for a simplified workflow. We propose the use of HL60 cells (suspension cells) that will show the necessary sensitivity to ETC dysfunction.

2. On the day of the experiment, pre-heat plate incubator/warmer and reader to 37°C.

3. Solution Preparation
   a. Pre-heat the Mito-ID® HS Oil to 37°C. Pre-warm RPMI media.

   b. Prepare the Mito-ID® Extracellular O₂ Sensor Probe Solution by reconstituting the Mito-ID® Extracellular O₂ Sensor Probe in 1ml of sterile, nuclease-free water. Gently mix to make solution homogenous.

   c. Prepare O₂ Sensor Probe Assay Mix

   d. Using a 1:14 dilution factor, dilute a sufficient volume of the Mito-ID® Extracellular O₂ Sensor Probe Solution with pre-warmed RPMI media. Mix gently and well.

   e. Prepare the Mito-ID® Extracellular pH Probe Solution. Once the provided probe has warmed to room temperature (or 37°C) add 1ml of sterile and nuclease-free water. Mix gently and well to ensure homogenous mixture.
f. Prepare Respiration Buffer using the following formulation; 1mM K-phosphate, 20mM Glucose, 0.07M NaCl and 0.05M KCl, 0.8mM MgSO₄, 2.4mM CaCl₂, pH 7.4

g. Prepare pH Assay Mix by diluting the Mito-ID® pH Extracellular Probe Solution using a 1:14 dilution factor with the freshly prepared Measurement Buffer.

Overview of Setup

The Mito-ID® Extracellular O₂ Sensor Probe and Mito-ID® pH Extracellular Probe (ENZ-51048) can be multiplexed or used in parallel (as shown). This will allow for simultaneous glycolytic flux and ETC analysis. Compound / drug of interest should be serially diluted.

1. Prepare Compound/drug serial dilution for assay

   **NOTE:** Compound/drug of interest should be prepared separately for both O₂ and pH analysis when used in parallel.

   O₂ Detection- Drug will be diluted to the desired concentration using the O₂ Sensor Probe Assay Mix (prepared from step 2.c), pH Detection- Drug will be diluted to the desired concentration using the pH Assay Mix (prepared from step 2.f).

2. Add to each well 70µL of the O₂ Sensor Probe Assay Mix containing the compound/drug of interest or the pH Assay Mix-containing the compound/drug of interest.

3. Set plate aside on temperature block and load 5µL of the HL60 cells (or cell choice).
NOTE: HL60 cells are added to a final concentration of ~4.0x10^6 cells/ml and ~1.75x10^6 cells/ml for O₂ and pH detection, respectively. Optimization will be required for cell line used.

4. Measurement

Read and compare measurements.

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 Mito-ID® Extracellular O₂ Consumption Assay (ENZ-51045) and Mito-ID® Extracellular pH Sensor Probe (ENZ-51048), show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP).
Mitochondrial toxicity has been implicated as a major contributor to drug-induced toxicity. This is not surprising when one considers both the multitude of sites where mitochondrial function can be perturbed. Detection of drug-induced toxicity in cells is wholly unsuitable when using conventional methods, since cells are capable of energetically circumventing mitochondrial insult by increasing ATP production via glycolysis. Standard cell viability assays are blind to such mitochondrial perturbation.

The Mito-ID® Extracellular O₂ Sensor (HS) kit and the Mito-ID® Extracellular pH Sensor Probe provides electron transport chain and glycolytic flux analysis. Data may be assessed by using endpoint detection or by assessing the rate change of the probe signal in relation to the drug concentration used. True mitochondrial toxicity is expected to result in a decrease in oxygen consumption and an increase in acidification as a result of glycolytic compensation. Following drug treatment, a decrease in both oxygen consumption and extracellular acidification indicates non-specific mitochondria toxicity. These expected results are represented below.

Schematic representation of metabolic response to drug treatment
REFERENCES

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