MITO-ID® Extracellular pH Sensor Kit

Catalog #: ENZ-51048
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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.
INTRODUCTION AND ASSAY PRINCIPLE

Metabolic changes can play a critical role in a variety of disease states and toxicities. Insight into the two main cellular ATP generating pathways; glycolysis and oxidative phosphorylation, therefore is particularly informative when examining metabolic perturbations. MITO-ID® Extracellular pH Sensor Kit is used for the assessment of extracellular acidification. As lactate production is the main contributor to this acidification, Enzo’s pH Sensor Probe is a convenient and informative measure of cellular glycolytic flux. Such analysis of glycolytic activity helps with assessing alterations in glucose metabolism, detecting glycolytic inhibition and be used to detect mitochondrial dysfunction. The fluorescent pH-sensitive probe and ratiometric time resolved fluorescence detection system helps overcome the calibration and biocompatibility issues associated with some existing probes. Spectral compatibility with MITO-ID® Extracellular O₂ Sensor Probe (ENZ-51044 or ENZ-51045), facilitates a multiplexed measurement approach providing a comprehensive metabolic assessment of test cells.

ASSAY PRINCIPLE

The MITO-ID® Extracellular pH Sensor Probe is an easy mix and measure, 96 or 384 well fluorescence plate reader based approach for the analysis of extracellular acidification. The reagent is chemically stable and inert, water soluble and cell impermeable, and exhibits a positive signal response (increased signal with increased acidification) across the biological range pH 6–7.5. MITO-ID® Extracellular pH Sensor Probe spectral response characteristics ensure it is the ideal choice for flexible, high throughput assessment of ECA / ECAR, overcoming many of the issues associated with the more complex potentiometric pH approach. Rates of extracellular acidification are calculated from changes in fluorescence signal over time, and, as the measurement is fully reversible, measurement of time courses and multiple drug treatments are possible.
KIT CONTENTS

- MITO-ID® Extracellular Sensor Probe (solid form)
- Respiration Buffer (1 Tablet) (1 mM K-Phosphate, 20 mM Glucose, 70 mM NaCl, 50 mM KCl, 0.8 mM MgSO_4, 2.4 mM CaCl_2)

STORAGE AND HANDLING

Store at 2-8°C. Do not freeze. Reconstituted product can be aliquoted and stored at -20°C. Use within one month and avoid freeze/thaw. Protect product from prolonged exposure to light.

STABILITY

1 year upon receipt.

FORMAT

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

OTHER MATERIALS NEEDED

1. Conventional or time-resolved fluorescence plate reader. (Excitation 340-410 nm or 535 nm) with an (Emission 590-690 nm)
2. Plate reader software (Recommended, VictorX4, FLUOstar, BMG, Perkin Elmer, Labtech)
3. Clear 96 well plate (Costar recommended)
4. Sterile, nuclease-free water
5. Tissue Culture hood and Incubator
6. Heating Block
7. Appropriate seeding material
8. Appropriate cell culture media
9. Additional reagents and material might be required depending on assay and conditions using.

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.
LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- 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 or reconstituting components.
- 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.
- Complete removal of all solutions and buffers during wash steps.
- 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.
MEASUREMENT PARAMETERS

The MITO-ID® Extracellular O₂ Sensor Probe is a chemically stable and inert, cell impermeable H⁺ sensing fluorophore.

![Graph showing excitation and emission spectra of MITO-ID® Extracellular pH Sensor Probe.](image)

**Fig. 1.** Excitation and Emission spectra of MITO-ID® Extracellular pH Sensor Probe. Left panel shows normalized excitation (Ex 340-410 nm; Peak 360-380 nm). Right panel show emission maxima (Em 590, 615 and 690 nm) fold increase between pH 6.0 and pH 7.5).

INSTRUMENT SETTINGS

Two fluorescence modalities can be optimally used with the MITO-ID® Extracellular pH Sensor Probe, depending on plate reader type and instrument setup.

**NOTE:** We strongly recommend only using fluorescence plate reader equipped with temperature control.

**Standard: TR-F Measurement**

Measurement using time resolved fluorescence (TR-F) provides flexibility to use a wide range of commonly available plate readers. TR-F measurement reduces non-specific background and increases probe sensitivity. Optimal delay time is ~100 µs and gate (integration) time is 100 µs.

**NOTE:** pH Sensor Probe should return a S:B ≥3.

**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 and to express ECA as a function of [H⁺].

**NOTE:** pH Sensor Probe should return a S:B ≥10.

Optimal dual-delay and gate (integration) times:

- Integration window 1: 100µs delay (D₁), 30µs measurement time (W₁)
- Integration window 2: 300µs delay (D₂), 30µs measurement time (W₂)
MITO-ID® Extracellular pH Sensor Probe may also be used in non TR-F Intensity mode on some plate readers, although we recommend running the Signal Optimization protocol to confirm an acceptable S:B, and optimizing cells seeding density.

User may see better performance using filter based plate readers.

**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 pH Sensor Probe, and can provide measurements of extracellular acidification that are more stable and with a wider dynamic range than measuring signal Intensity or standard TR-F. NOTE: S:B for Integration window 2 is recommended to be ≥10 to allow accurate Lifetime calculation.

![Diagram illustrating dual-read TR-F measurement](image)

**Fig. 2.** Illustrating dual-read TR-F measurement. Use the dual intensity readings to calculate the corresponding Lifetime (µs) using the following transformation: 

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

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.

**NOTE:** Lifetime values should be in the range ~200 µs for cells assayed in respiration buffer at approx. pH 7.4, increasing up to >400µs upon acidification, and should only be calculated from samples containing pH 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 Respiration Buffer tablet in 50 mL of water, warm to assay temperature (37°C), pH adjust to approx. pH7.4 and filter sterilize using a 0.22μm filter.

**STEP 2:** Reconstitute contents of the MITO-ID® Extracellular pH Sensor Probe in 1 mL of respiration buffer, gently aspirating 3-4 times.

**STEP 3:** Prepare 8 replicate wells of a 96-well plate, by adding 150 μL pre-warmed respiration buffer to each well (A1-A4, B1-B4).

**STEP 4:** Add 10 μL reconstituted MITO-ID® Extracellular pH Sensor Probe to 4 of the replicate wells (A1-A4) and 10 μL respiration buffer to the remaining replicates wells (B1-B4).

**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>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>A</td>
<td>Respiration Buffer + pH Sensor Probe</td>
<td>Respiration Buffer + pH Sensor Probe</td>
<td>Respiration Buffer + pH Sensor Probe</td>
<td>Respiration Buffer + pH Sensor Probe</td>
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<tr>
<td>B</td>
<td>Respiration Buffer</td>
<td>Respiration Buffer</td>
<td>Respiration Buffer</td>
<td>Respiration Buffer</td>
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</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 a guideline. Optimization due to cell line of choice or conditions might be required.

**NOTE:** PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Buffers and media should be prepared freshly on the day of the experiment. Allow provided probe to warm to room temperature for about 5-10 minutes. Upon heating to room temperature, briefly centrifuge the MITO-ID® Extracellular pH Sensor Probe vial to gather the contents at the bottom of the tube. Typical assay will be performed at 30°C.
CELL CULTURE AND PLATING

For Adherent cells, seed cells in a 96-well plate at a density (typically 30,000–80,000 cells/well) in 200 µL culture medium. Incubate overnight in a CO₂ incubator at 37°C.

TIP: Prepare a cell titration experiment to identify optimum cell seeding density for a specific cell type.

For Suspension cells, seed on the day of assay in 150 µL respiration buffer at a density of ~250,000–500,000 cells / well.

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

REAGENT PREPARATION

Reconstitute Respiration Buffer tablet in 50 mL of water, warm to assay temperature (37°C), pH adjust to approx. pH7.4 and filter sterilize using a 0.22 µm filter.

Reconstitute the contents of the MITO-ID® Extracellular pH Sensor Probe in 1 mL of respiration buffer, gently aspirating 3-4 times.

Prepare test compounds, controls and dilutions as desired. Typical controls are oxamic acid (inhibitor; decrease ECA), FCCP (ETC uncoupler; increases ECA) and glucose oxidase (GOx; signal control).

NOTE: We recommend that all culture media and stock solutions to be used in the assay be 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.

ASSAY PROCEDURE

To assess Extracellular Acidification (ECA) or to investigate the effect of a treatment on glycolytic flux, 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 wash cells twice, using 100 µL of respiration buffer per well for each wash. After removing the second wash, replace with 150 µL of fresh respiration buffer. NOTE: We recommend always leaving two wells (H11 and H12) free from the addition of MITO-ID® Extracellular pH Sensor Probe, for use as Blank Controls. Add 150 µL of respiration buffer to these Blank Control wells.

STEP 2: Add 10 µL reconstituted pH Sensor Probe to each well, except those wells for use as Blank Controls. Add 10 µL respiration buffer to these Blank Control wells. NOTE: If plating a full 96 well plate of assays, we recommend simplifying Step 1 and 2 by preparing a stock solution
containing the 1 mL of reconstituted pH Sensor Probe added to 15 mL pre-
warmed respiration buffer, and using a multi-channel pipette to add 150 µL of this diluted pH Sensor Probe stock to each well. Add 150 µL of respiration buffer only to each Blank Control well.

STEP 3: Test compound stock or vehicle (typically 1-10 µL) 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: Read the plate immediately in a fluorescence plate reader. The plate should be measured kinetically for >120 minutes. When the measurement is completed, remove the plate and save measured data to file.

**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 EXTRACELLULAR ACIDIFICATION**

Plot the Blank Control well corrected MITO-ID® Extracellular pH Sensor Probe Intensity or Lifetime values versus Time. Select linear portion of the signal profile (avoiding any initial lag or subsequent plateau) and apply linear regression to determine the slope (ECA) and correlation coefficient for each well.

**NOTE:** This approach is preferable to calculating a slope from averaged profiles.

![Fig. 3. Typical lifetime profile of MITO-ID® Extracellular pH Sensor Probe for adherent cells, treated with typical control compounds, including oxamic acid](image)
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 dose 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.

**TITRATION OF CELL SEEDING DENSITY**

To determine an optimal cell seeding density for performing the MITO-ID® Extracellular pH Sensor Probe, for new cell types, seed replicate wells with a range of seeding densities (typically 0, 10,000, 20,000, 40,000, 60,000 and 80,000 cells / well). Plot the data generated as a function of intensity or lifetime values versus time.

![Graph showing extracellular pH profiles](image)

**Fig. 4.** Extracellular Acidification rate profiles (ECA) are shown for A549 cells seeded at 0, 10,000, 20,000, 40,000, 60,000 and 80,000 cells / well. In this experimental example, a seeding density of 40,000 cells / well was chosen for study as this provided a suitable balance between ECA response and cell availability.

**CELLULAR ENERGY FLUX ASSAY**

Multiparametric (or multiplex) combination of MITO-ID® Extracellular pH Sensor Probe together with MITO-ID® Extracellular O₂ Sensor Kit (High Sensitivity) allows the simultaneous real time measurement of glycolysis and mitochondrial respiration, and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways.
Fig. 5. 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® O₂ Extracellular Sensor Kit (High Sensitivity) and MITO-ID® Extracellular pH Sensor Probe, show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP).

**CALIBRATION OF MITO-ID® EXTRACELLULAR PH SENSOR PROBE TO A [H⁺] SCALE**

It is possible to express Extracellular Acidification (ECA) as a function of pH [H⁺] versus time. This is achieved by first creating a calibration standard curve, by measuring TR-F intensity or preferably Lifetime values (selecting stabilized readings over a 30 minute read), from a range of pH buffered standards at the appropriate assay temperature. Select the linear portion of the standard curve and apply linear regression to determine the calibration function.
Fig. 6. MITO-ID® Extracellular pH Sensor Probe calibration in Lifetime scale at 30°C and 37°C using pH buffered PBS at increments of 0.2 across a pH range 6.0–7.5.

REFERENCES

## Instrument and Measurement Settings

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Optical Configuration</th>
<th>Integ 1 (D1/W1) Integ 2 (D2/W2)</th>
<th>Optimum Mode*</th>
<th>Ex (nm) Em (nm)</th>
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<tbody>
<tr>
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<td>100 / 30 μs 300 / 30 μs</td>
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