Product Manual

MITO-ID® Fatty Acid Oxidation Assay

ENZ-KIT184 **MITO-ID® Fatty Acid Oxidation Assay**

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Product Manual

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.

TABLE OF CONTENTS

Introduction 2
Applications 2
Kit Contents and Storage 2
Stability 3
Format 3
Required and Optional Materials 3
Precautions 3
Limitations 3
Technical Hints 4
Methods and Procedures 4
- Cell Culture and Plating 5
- Reagent Preparation 5
- Assay Procedure 5

Analysis 7
- Sample FAO-Driven Oxygen Consumption 7
- Evaluating Exogenous and Endogenous FAO 7
- Metabolic Characterization 7

References 8
Notes 8
Contact Information 9

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, neuro degeneration and ischemia. A major mechanism of drug-induced toxicity has been linked to mitochondria dysfunction. Fatty acid oxidation (FAO) is the primary metabolic pathway in a variety of tissues, becoming particularly important during periods of glucose deprivation. In organs FAO can provide greater than 75% of cellular ATP and is known as a key factor in cancer metabolism.

The MITO-ID® Fatty Acid Oxidation Assay (ENZ-KIT184) in combination with the MITO-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045) provides a highly flexible, 96-well, fluorescence plate reader based approach, for the direct, real time analysis for the measurement of FAO-driven respiration.

This kit is designed for use with MITO-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045) and contains the 18C unsaturated fatty acid Oleate as substrate, supplied as a 2:1 BSA conjugate. For convenient preparation of measurement media this kit also contains a buffer tablet and L-Carnitine and two FAO modulators, Etomoxir and FCCP.

APPLICATIONS

- Determine FAO-driven Oxygen Consumption
- Evaluate Exogenous and Endogenous FAO
- Metabolic Characterization

NOTE: The MITO-ID® Fatty Acid Oxidation Assay (ENZ-KIT184), a companion with for use with the MITO-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045), is recommended for cell-based applications

KIT CONTENTS & STORAGE

Kit #	Reagent Description	Volume	Quantity	Storage	# of tests
ENZ-KIT184	Oleate-BSA Conjugate	1 mL	1 vial	+4°C	200
	BSA Control	0.5 mL	1 vial	+4°C	100
	Base Measurement Media Tablet (KHB)	Tablet	1 tablet	RT	200
	L-Carnitine	4 mg	1 vial	+4°C	200
	FCCP	0.004 mg	1 vial	-20°C	55
	Etomoxir	0.074 mg	1 vial	-20°C	55

STABILITY

12 months upon receipt

FORMAT

Standard 96-well plates.

REQUIRED AND OPTIONAL MATERIALS

1. Repeater pipette or multiple-channel pipette
2. Plate heater
3. MITO-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045)
4. Standard 96-well TC+ plates or 96-well black wall clear bottom TC+ plates.
5. Cells or isolated mitochondria
6. Cell culture medium (for cells)
7. Measurement buffer (for isolated mitochondria)
8. Compounds of interest
9. Fluorescence plate reader
10. Mitochondrial substrate (succinate, glutamate or malate) (for isolated mitochondria assay)
11. ADP (for isolated mitochondria assay)
12. Components for glucose deprivation media (Glucose free DMEM, glucose, L-glutamine, foetal bovine serum, pen/strep)

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.
- 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.
- 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.

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.

NOTE: *PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Before starting the procedure, ensure that measurement settings and cell number have been optimized as per the MITO-ID® Extracellular O_2 Sensor Kit (High Sensitivity) (ENZ-51045) user manual.*

CELL CULTURE AND PLATING

Count cells and adjust to the desired plating density in culture medium (typically ~6x10^4 cells per well for 2D cultures). Return the plate for culture overnight (typically >14 hours).

REAGENT PREPARATION

Prepare Kit Components:

- L-Carnitine: Dissolve vial contents in 400 µL (100X) of sterile distilled water to prepare 50 mM stock. Aliquot in 100 µL volumes and store at -20°C, each aliquot is sufficient for 100 wells.
- FFCP: Dissolve vial contents in 60 µL (100X) of DMSO to prepare 250 µM stock. Aliquot in 20 µL volumes and store at -20°C, each aliquot is sufficient for 20 wells.
- Etomoxir: Dissolve vial contents in 550 µL (10X) of sterile distilled water to prepare 400 µM stock. Aliquot in 50 or 100 µL volumes and store at -20°C, each 100 µL aliquot is sufficient for 10 wells.
- Base Glucose Deprivation Media (optional): Glucose-free DMEM, 1 mM glucose, 1.0 mM L-glutamine, 1% FBS, 100 U/mL/0.1 mg/mL Pen/Strep. Media can be stored for 2 weeks at +4°C.
- Base Measurement Media: Dissolve kit buffer tablet in 100 mL of distilled water, warm to 37°C, bring to pH 7.4 using HCl and NaOH and filter sterilize. Media can be stored for 3 weeks at +4°C.

Prepare on Day of Measurements:

- Glucose Deprivation Media (optional): To Base Glucose Deprivation Media, add 0.5 mM L-Carnitine (1/100 of stock).
- FA-Free Measurement Media: To Base Measurement Media, add 0.5 mM L-Carnitine (1/100 dilution of stock).
- FA Measurement Media: FA-Free Measurement Media + 150 mM Oleate-BSA.

ASSAY PROCEDURE

To facilitate the measurement of fatty acid oxidation-driven respiration.

NOTE: *We recommend the use of triplicate wells for each treatment.*

STEP 1: Wash cells by placing the pate on a plate block heater set to assay temperature and remove spent culture media using an

aspirator, being careful not to dislodge cells form the base of the wells. Using a multichannel or repeater pipette, add 100 µL of the pre-warmed FA-Free Media to each well. Repeat wash step 1X.

STEP 2: Add 90 µL of pre-warmed FA Measurement Media to each well except those wells being used as FA-Free controls. To these add 85 µL of FA-Free Measurement Media and 5 µL of BSA control.

NOTE: *FA-Free Measurement Media is used as a control to measure O2 consumption without exogenous Oleate-BSA. BSA control is added to ensure that the free concentrations of test compounds are consistent between FA and FA-Free conditions. The BSA concentration used in FA-free control wells should be consistent with the BSA concentration in samples containing Oleate-BSA (Oleate-BSA is a 2:1 conjugate).*

STEP 3: Add 10 µL of reconstituted MITO-ID® Extracellular O_2 Sensor Probe reagent to each well, except those wells for use as Blank Controls. Add 10 µL of FA Measurement Media to Blank Control wells.

NOTE: *If measuring a full 96-wel plate , we recommend combining Steps 1 and 2 by diluting reconstituted MITO-ID® Extracellular O_2 Sensor Probe stock 1 in 10 in the relevant measurement media and, using a multichannel pipette, to add 100 µL to each well. Add 100 µL of FA-Measurement Media (no MITO-ID® Extracellular O_2 Sensor Probe) to the Blank Control wells.*

STEP 4: The MITO-ID® Fatty Acid Oxidation Kit can be used for metabolic characterization or to determine the impact of cell treatment (drug treatment, signaling pathway modulation, genetic manipulations etc.).

Metabolic Characterization: Cellular dependence on, or preference for FAO can be determined using kit control compounds Etomoxir and FCCP in the presence and absence of added Oleate. For compound addition, add 10 µL of Etomoxir and 1 µL FCCP stock to designated wells. Etomoxir blocks LCFA uptake, while FCCP increases cellular energy demand thereby increasing FAO dependence.

NOTE: *Ensure a minimum of 10 minutes between Etomoxir addition and assay measurement.*

Cell Treatment: Various cell treatments can be performed including genetic manipulation, signaling pathway modulation and drug treatment. Drug screening is used here as an exemplar application: Add test compound or vehicle (typically 1-5 µL) to test wells. Measurement is typically performed both in the presence and absence of Oleate-BSA. Additional BSA control stock is added to wells without Oleate, 6-8 point compound dilutions are typically

used. Cells are co-treated with FCCP if impact on maximal FAO is being determined. Etomoxir is used as a control.

NOTE: *We recommend keeping volume of added compound low to minimize potential vehicle effects.*

STEP 5: Seal each well with 100 µL of pre-warmed HS Mineral Oil, taking care to avoid bubbles. Read the plate immediately in a fluorescent plate reader as described in the MITO-ID® Extracellular O_2 High Sensitivity Kit.

ANALYSIS

SAMPLE FAO-DRIVEN OXYGEN CONSUMPTION

Untreated cells show a steady signal increase reflecting ETC-driven oxygen consumption. Signal control shows prove signal in the absence of cell respiration. Etomoxir treatment prevents Oleate import, resulting in reduced availability of reducing equivalents and resultant decrease in ETC activity. The remaining ETC activity is driven by metabolic activity other than LC FAO. FCCP treatment induces maximal ETC activity by dissipating the mitochondrial membrane potential increased demand for reducing equivalents causes a concomitant increase in FAO as indicated by the rapid increase in signal. This strong increase in ETC activity is not observed where exogenous LCFA is unavailable or where import is inhibited.

EVALUATING EXOGENOUS AND ENDOGENOUS FAO

FAO-driven respiratory activity can be interrogated further by calculating he rate of signal change for each FAO assay profile, facilitating assessment of exogenous FAO (Oleate supplied), endogenous FAO (Oelate-free) and non-LC FAO (Etomoxir treated). This can be determined using slopes (m) calculated from the linear portion of each profile:

- Exogenous FAO = $m_{Oleate} - m_{Etomoxir}$
- Endogenous FAO = $m_{Oleate\text{-}free} - m_{Etomoxir}$
- Non-LC FAO = $m_{Etomoxir} - m_{Signal\ Control}$

METABOLIC CHARACTERIZATION

The FAO kit facilitates interrogation of oxygen consumption due to exogenous FA substrates such as Oleate, endogenous FA stores, and non-long chain FA substrates. The impact of differentiation on C2C12 cell metabolism can be determined using this approach. Measuring maximal respiratory capacity of fully confluent C2C12

myoblasts (undifferentiated), and multinucleated myotubes (differentiated), and the proportion of this capacity driven by long chain FAO. Differentiation from myoblasts to multinucleated myotubes significantly increases maximal respiratory capacity, measuring after a 1 hour glucose deprivation step. This is driven by both endogenous substrates and an increased capacity to metabolize exogenous FA substrates (Oleate-BSA) to meet an increased demand for reducing equivalents imposed by FCCP treatment.

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NOTES

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