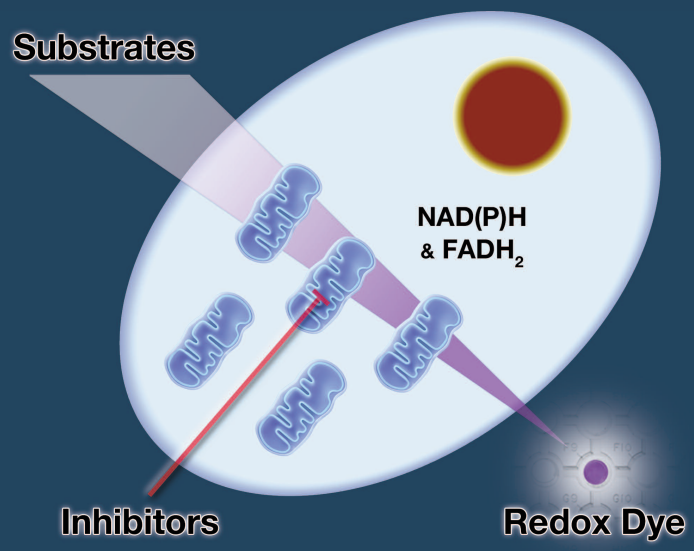
MitoPlate™ Technology

Analyzing Mitochondria at Higher Resolution

New Probes of Mitochondrial Function

MitoPlates from Biolog provide a powerful new research tool by allowing scientists to run preconfigured sets of 96 mitochondrial function assays in one experiment. Mitochondria can be interrogated and characterized in novel ways, looking at rates of substrate metabolism, sensitivity to drugs and other chemicals, and effects of mutations in mitochondria-related genes.



Substrates or inhibitors permeate the cell membrane and enter mitochondria, stimulating or inhibiting production of NAD(P)H or $FADH_2$ which is then measured using a tetrazolium redox dye.

Investigate how mitochondria change with:

- *Cell differentiation*
- *Cancer & ageing*
- *Neurological disorders*
- *Metabolic disorders*
- *Immune cell activation*
- *Bacterial/viral infection*
- *Inborn genetic defects*

Assay Principle

Mitochondrial function is assayed by measuring the rates of electron flow into and through the electron transport chain from metabolic substrates that produce NAD(P)H or $FADH_2$ such as L-malate, succinate, pyruvate, etc. Each substrate follows a different route, using different transporters to enter the mitochondria and different dehydrogenases to produce NAD(P)H or $FADH_2$, The electrons travel from the beginning (complex 1 or 2) to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor that turns purple upon reduction. Additional MitoPlate assays probe the sensitivity of the mitochondria to a set of 22 diverse inhibitors.

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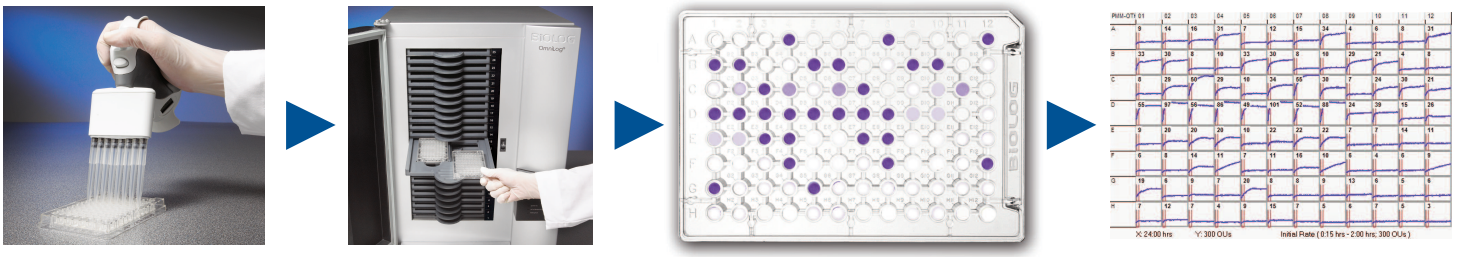
MitoPlate S-1 with 30 Substrates:

No Substrate Control	α-D-Glucose	Glycogen	D-Glucose-1-PO4
D-Glucose-6-PO4	D-Gluconate-6-PO4	D,L-α-Glycerol-PO4	L-Lactic Acid
Pyruvic Acid	Citric Acid	D,L-Isocitric Acid	cis-Aconitic Acid
α-Keto-Glutaric Acid	Succinic Acid	Fumaric Acid	L-Malic Acid
α-Keto-Butyric Acid	D,L-β- Hydroxy-Butyric Acid	L-Glutamic Acid	L-Glutamine
Ala-Gln	L-Serine	L-Ornithine	Tryptamine
Sparker Malate Control	Acetyl-L-Carnitine	Octanoyl-L-Carnitine	Palmitoyl-D,L-Carnitine
Pyruvic Acid	γ-Amino-Butyric Acid	α-Keto-Isocaproic Acid	L-Leucine

MitoPlate I-1 with 22 Inhibitors:

No Substrate Control	No Inhibitor Control	Meclizine
Complex I Inhibitor Rotenone	Complex I Inhibitor Pyridaben	Berberine
Complex II Inhibitor Malonate	Complex II Inhibitor Carboxin	Alexidine
Complex III Inhibitor Antimycin A	Complex III Inhibitor Myxothiazol	Phenformin
Uncoupler FCCP	Uncoupler 2,4-Dinitrophenol	Diclofenac
Ionophore, K Valinomycin	Calcium / $CaCl_2$	Celastrol
Gossypol	Nordihydro-guaiaretic acid	Trifluoperazine
Polymyxin B	Amitriptyline	Papaverine



Simple Assay Procedure:

STEP 1: Prepare and pipet assay mixture containing cell permeabilizing buffer and redox dye into appropriate wells.

STEP 2: Start the assays by adding 2x cell suspension to all wells.

STEP 3: Load the MicroPlate into the OmniLog® for kinetic reading of the rate of purple color formation.

Ordering Information:

Catalog #	Description
14104	MitoPlate I-1
14105	MitoPlate S-1
72303	Biolog MAS
74353	Biolog Redox Dye Mix MC
96161	OmniLog PM-M System (NA Plug)
96162	OmniLog PM-M System (Schuko Plug)
96164	OmniLog PM-M System (UK Plug)

Not Included: Saponin permeabilizing solution and substrate solutions for MitoPlate I-1. Please review Instructions for Use prior to ordering.

Unique Features and Advantages:

- MitoPlates are preloaded with 96 tests ready for use
- Plates designed to measure effects of substrates and inhibitors on mitochondrial function
- Easy, robust protocols with any cell type – adherent or suspension cells, transformed cell lines or primary cells
- Assays need only 20,000 to 40,000 cells per well
- Novel tetrazolium dye chemistry provides a terminal electron acceptor in easy to read colorimetric assays
- OmniLog instrument provides automated temperature controlled incubation and kinetic reading of multiple plates (50 plates at 15 min, or 16 at 5 min intervals)

OmniLog System and Analysis Software

The OmniLog Instrument and associated software allows for real-time recording and kinetic analysis of electron flow rates. The OmniLog can simultaneously incubate and read up to 50 MitoPlates and provides powerful analysis tools to get the most from your experimental data.

Part 00A 069, Rev C 1/2018

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New Mitochondrial Function Assay Technology

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Biolog, Inc. Hayward, CA, USA

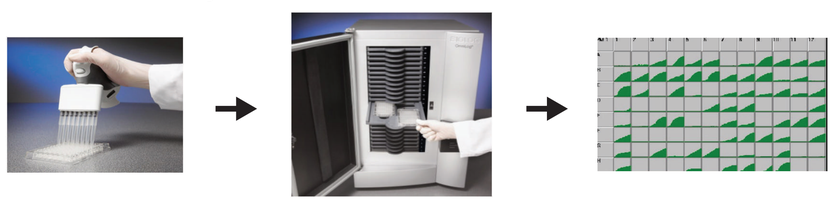
Abstract

We have developed a new mitochondrial function assay technology that measures the rates of metabolism of mitochondrial substrates and the sensitivity of metabolism of these substrates to mitochondrial inhibitors. The technology employs saponin permeabilized cells and a redox dye added to 96-well microplates that contain mitochondrial substrates or inhibitors precoated and dried into the wells. The MitoPlate S-1™ has a triplicate repeat of a set of 31 substrates. Mitochondrial function is assayed by measuring the rates of dye reduction from electrons flowing into and through the electron transport chain from substrates whose oxidation produces NADH (e.g., L-malate) or $FADH_2$ (e.g., succinate). The electrons donated to complex 1 or complex 2 travel to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor and changes from colorless to a purple formazan upon reduction. All 96 assays in the MitoPlate are run concurrently, and each assay provides different information because each substrate follows a different metabolic route using different transporters to enter the mitochondria, and then different dehydrogenases to produce NADH or $FADH_2$. The MitoPlate S-1™ can also be used to assess the activity and specificity of substrate transport inhibitors, dehydrogenase inhibitors, or electron transport chain inhibitors. A second assay plate, the MitoPlate I-1™, provides another assessment of mitochondrial function by measuring the sensitivity of mitochondrial electron flow to a set of 22 diverse inhibitors titrated at 4 dilutions. The I-1 plates can be run using any of the NADH or $FADH_2$ producing substrates, each providing additional information. Using these new assays we show that the mitochondria from different cell types exhibit different functional properties. This new technology will assist efforts to understand how mitochondria change in cell models of human disorders that have a mitochondrial basis.

The Assay Technology

Figure 1 outlines the simple assay protocol. 30 µl of a permeabilizing assay mix containing saponin and Redox Dye MC in isotonic buffer is pipetted into all wells and incubated at 37º C for 1 hour. To start the assay, 30 ul of a cell suspension in isotonic buffer is added to each well. The recommended cell density is 1,000,000 cells/ml resulting in 30,000 cells/well. To record the rates of dye reduction in the wells, the MitoPlate is loaded into the OmniLog, which reads at 5 minute intervals for 2 to 4 hours. For MitoPlate I-1 with 22 mitochondrial inhibitors, the permeabilizing assay mix also contains an NADH or $FADH_2$ producing substrate such as L-malate or succinate. **Figure 3 and 4** show, respectively, the test layout in the MitoPlate S-1 and the MitoPlate I-1.

Figure 1. The Assay Protocol.



Assays and Results

Figure 2. With this assay technology, mitochondrial function is profiled in a new way by measuring the rates of dye reduction from electrons flowing into and through the electron transport chain from substrates whose oxidation produces NADH or $FADH_2$.

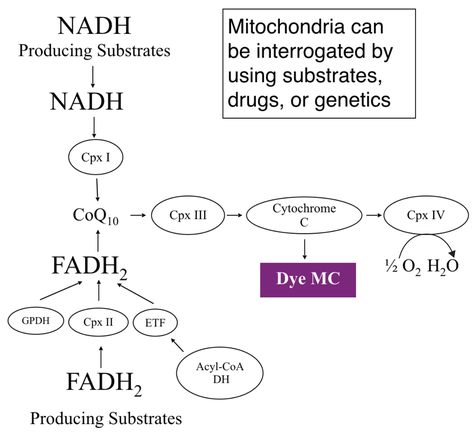


Figure 3. The MitoPlate S-1 simultaneously assays the metabolic rates of potential NADH or $FADH_2$ producing substrates.

MitoPlate S-1: Mitochondrial Function Assays Testing Substrates

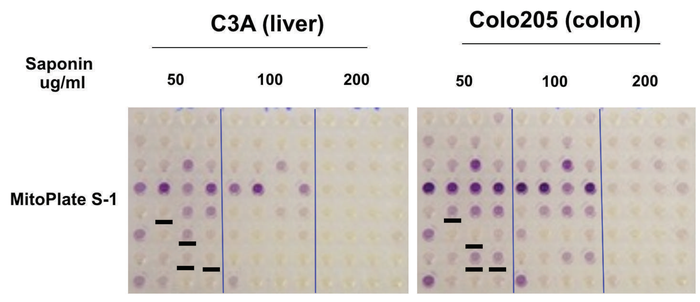
A1 No Substrate	A2 α-D-Glucose	A3 Glycogen	A4 D-Glucose-1-PO4	A5 No Substrate	A6 α-D-Glucose	A7 Glycogen	A8 D-Glucose-1-PO4	A9 No Substrate	A10 α-D-Glucose	A11 Glycogen	A12 D-Glucose-1-PO4
B1 D-Glucose-6-PO4	B2 D-Gluconate-6-PO4	B3 D,L-α-Glycerol-PO4	B4 L-Lactic Acid	B5 D-Glucose-6-PO4	B6 D-Gluconate-6-PO4	B7 D,L-α-Glycerol-PO4	B8 L-Lactic Acid	B9 D-Glucose-6-PO4	B10 D-Gluconate-6-PO4	B11 D,L-α-Glycerol-PO4	B12 L-Lactic Acid
C1 Pyruvic Acid	C2 Citric Acid	C3 D,L-Isocitric Acid	C4 cis-Aconitic Acid	C5 Pyruvic Acid	C6 Citric Acid	C7 D,L-Isocitric Acid	C8 cis-Aconitic Acid	C9 Pyruvic Acid	C10 Citric Acid	C11 D,L-Isocitric Acid	C12 cis-Aconitic Acid
D1 α-Keto-Glutaric Acid	D2 Succinic Acid	D3 Fumaric Acid	D4 L-Malic Acid	D5 α-Keto-Glutaric Acid	D6 Succinic Acid	D7 Fumaric Acid	D8 L-Malic Acid	D9 α-Keto-Glutaric Acid	D10 Succinic Acid	D11 Fumaric Acid	D12 L-Malic Acid
E1 α-Keto-Butyric Acid	E2 D,L-β-Hydroxy-Butyric Acid	E3 L-Glutamic Acid	E4 L-Glutamine	E5 α-Keto-Butyric Acid	E6 D,L-β-Hydroxy-Butyric Acid	E7 L-Glutamic Acid	E8 L-Glutamine	E9 α-Keto-Butyric Acid	E10 D,L-β-Hydroxy-Butyric Acid	E11 L-Glutamic Acid	E12 L-Glutamine
F1 Ala-Gln	F2 L-Serine	F3 L-Ornithine	F4 Tryptamine	F5 Ala-Gln	F6 L-Serine	F7 L-Ornithine	F8 Tryptamine	F9 Ala-Gln	F10 L-Serine	F11 L-Ornithine	F12 Tryptamine
G1 L-Malic Acid 100uM	G2 Acetyl-L-Carnitine + L-Malic Acid 100uM	G3 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G4 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM	G5 L-Malic Acid 100uM	G6 Acetyl-L-Carnitine + L-Malic Acid 100uM	G7 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G8 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM	G9 L-Malic Acid 100uM	G10 Acetyl-L-Carnitine + L-Malic Acid 100uM	G11 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G12 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM
H1 Pyruvic Acid + L-Malic Acid 100uM	H2 γ-Amino-Butyric Acid + L-Malic Acid 100uM	H3 α-Keto-Isocaproic Acid + L-Malic Acid 100uM	H4 L-Leucine + L-Malic Acid 100uM	H5 Pyruvic Acid + L-Malic Acid 100uM	H6 γ-Amino-Butyric Acid + L-Malic Acid 100uM	H7 α-Keto-Isocaproic Acid + L-Malic Acid 100uM	H8 L-Leucine + L-Malic Acid 100uM	H9 Pyruvic Acid + L-Malic Acid 100uM	H10 γ-Amino-Butyric Acid + L-Malic Acid 100uM	H11 α-Keto-Isocaproic Acid + L-Malic Acid 100uM	H12 L-Leucine + L-Malic Acid 100uM

Figure 4. The MitoPlate I-1 simultaneously assays the sensitivity of NADH or $FADH_2$ producing pathways to 22 mitochondrial inhibitors.

MitoPlate I-1: Mitochondrial Function Assays Testing Inhibitors

A1 No Inhibitor No substrate With Saponin	A2 No Inhibitor No substrate With Saponin	A3 No Inhibitor No substrate With Saponin	A4 No Inhibitor No substrate With Saponin	A5 No Inhibitor With substrate With Saponin	A6 No Inhibitor With substrate With Saponin	A7 No Inhibitor With substrate With Saponin	A8 No Inhibitor With substrate With Saponin	A9 Mecllizine 1	A10 2	A11 3	A12 4
B1 Complex I Inhibitor Rotenone 1	B2 2	B3 3	B4 4	B5 Complex I Inhibitor Pyridaben 1	B6 2	B7 3	B8 4	B9 Berberine 1	B10 2	B11 3	B12 4
C1 Complex II Inhibitor Malonate 1	C2 2	C3 3	C4 4	C5 Complex II Inhibitor Carboxin 1	C6 2	C7 3	C8 4	C9 Alexidine 1	C10 2	C11 3	C12 4
D1 Complex III Inhibitor Antimycin A 1	D2 2	D3 3	D4 4	D5 Complex III Inhibitor Myxothiazol 1	D6 2	D7 3	D8 4	D9 Phenformin 1	D10 2	D11 3	D12 4
E1 Uncoupler FCCP 1	E2 2	E3 3	E4 4	E5 Uncoupler 2,4 Dinitrophenol 1	E6 2	E7 3	E8 4	E9 Diclofenac 1	E10 2	E11 3	E12 4
F1 Ionophore, K Valinomycin 1	F2 2	F3 3	F4 4	F5 Calcium CaCl2 1	F6 2	F7 3	F8 4	F9 Celastrol 1	F10 2	F11 3	F12 4
G1 Gossypol 1	G2 2	G3 3	G4 4	G5 Nordihydro-guaiaretic acid 1	G6 2	G7 3	G8 4	G9 Trifluoperazine 1	G10 2	G11 3	G12 4
H1 Polymyxin B 1	H2 2	H3 3	H4 4	H5 Amitriptyline 1	H6 2	H7 3	H8 4	H9 Papaverine 1	H10 2	H11 3	H12 4

Figure 5. Colon and liver cells were assayed for substrate metabolism using the MitoPlate S-1. Four major differences were found in their metabolism.



Mitochondrial substrate	C3A	Colo205
b-hydroxy-butyrate (E-2)	neg	pos
L-ornithine (F-3)	pos	neg
octanoyl-carnitine (G-3)	neg	pos
palmitoyl-carnitine (G-4)	neg	pos

Figure 6. Colon cells were assayed in MitoPlate I-1 with L-malate as the substrate. The cells were sensitive to Complex 1 and 3 inhibitors, but not Complex 2 inhibitors.

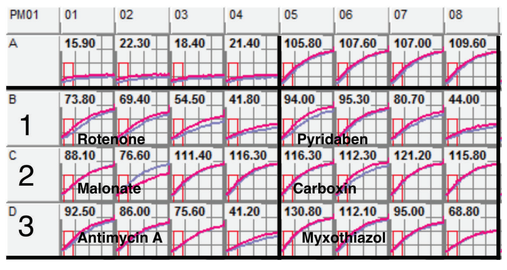
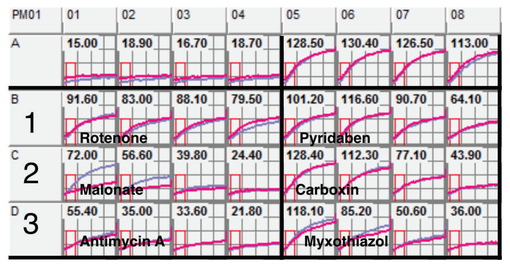


Figure 7. Colon cells were assayed in MitoPlate I-1 with succinate as the substrate. The cells were sensitive to Complex 2 and 3 inhibitors, but not Complex 1 inhibitors.



Conclusions

The MitoPlate assay technology enables profiling of mitochondrial function in much greater detail. The MitoPlates have 53 phenotypic assays already dried in the wells, so they can be tested at the same time by simply inoculating with a cell suspension. The assays are colorimetric and can be performed using any kinetic microplate reader. The technology provides a simple and highly sensitive discovery tool for mitochondrial researchers.

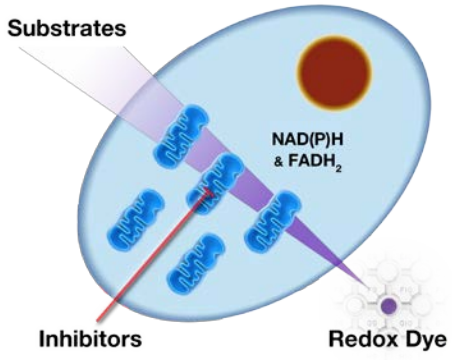
MitoPlate™ S-1 and MitoPlate™ I-1

for Characterization of Mammalian Cell Mitochondria

Assays: Mitochondrial Substrate Metabolism
Sensitivity to Mitochondrial Inhibitors

PRODUCT DESCRIPTIONS AND INSTRUCTIONS FOR USE

MitoPlate S-1 Cat. #14105
MitoPlate I-1 Cat. #14104



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The most current version of this Technical Bulletin can be downloaded from Biolog's website at www.biolog.com. Questions about the use of this product should be directed to Biolog, Inc. Technical Services by E-mail at tech@biolog.com.

For Research Use Only

Part# 00P 273, Rev C, October 2020

CONTENTS

I. Introduction

a. Overview

MitoPlates™ from Biolog provide a powerful research tool by allowing scientists to run preconfigured sets of 96 mitochondrial function assays in one experiment. Mitochondria can be interrogated and characterized in novel ways, looking at rates of substrate metabolism, sensitivity to drugs and other chemicals, and effects of mutations in mitochondrial genes.

b. Background

Mitochondria play a primary role in energy production by cells. It is clear that these organelles are dynamic as the quantity and structure of the mitochondria in cells can change. Mitochondria are complex, consisting of over 1,000 proteins, the vast majority of which are coded for by nuclear rather than mitochondrial DNA. In addition to proteins, mitochondria also have specialized membranes and they can interact with each other and with other cellular organelles such as endoplasmic reticuli.

c. Uses

By providing a new high resolution approach to assaying mitochondrial function, MitoPlates allow scientists to investigate how mitochondria change with differentiation, cancer and ageing, neurological disorders, metabolic disorders, immune cell activation, bacterial/viral infection, inborn genetic disorders, or any other change that can be experimentally modeled at the cellular level.

d. Advantages

MitoPlates provide a unique and powerful tool for simultaneously running 96 relevant assays to elucidate changes in mitochondrial properties. Some principal advantages are:

- **Proven Technology:** A sizeable volume of published literature documents the successful use of Biolog's PMM and MitoPlate technology as applied to mammalian cell research. An updated listing can be found in the Bibliography section of the Biolog website at

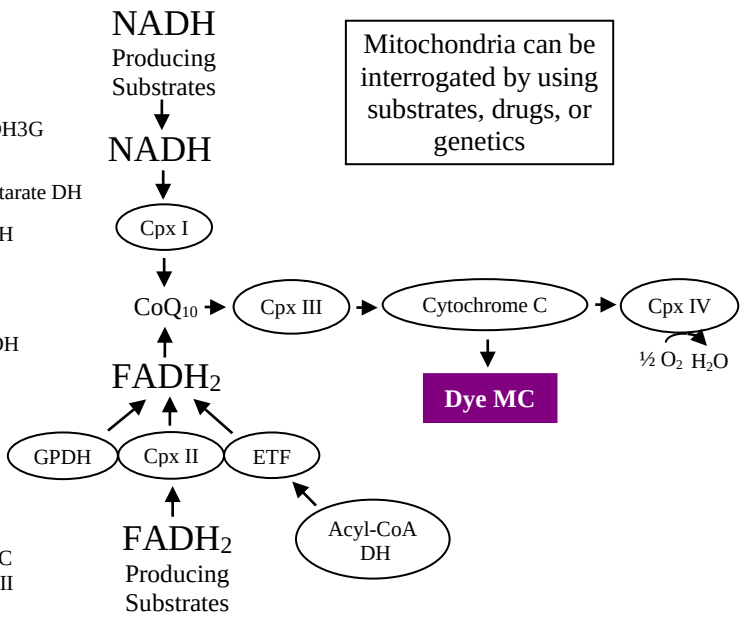
http://www.biolog.com/bibliography.php. The PMM technology is based upon measurement of live cell properties using a bioenergetics detection chemistry. The MitoPlates are an extension of PMM technology, performing similar bioenergetics detection, but on live cells that are instantaneously permeabilized to allow access to the mitochondrial organelles.

- **Simple Protocol:** Add the permeabilizing buffer with dye mix, add the cells, and read the rate of purple color formation. Purification of mitochondria is not required.
- **Fast Results:** For rate measurements, sufficient color forms in as little as two hours. The purple formazan product is soluble and stable and can be measured as soon as it forms.
- **Flexible Format:** The Biolog OmniLog® instrument is recommended for reading the MitoPlates because it can read multiple plates kinetically (up to 16 at 5 minute read intervals) while under temperature-controlled incubation. However, the MitoPlates can also be read with standard microplate readers that allow kinetic reading at OD_{590}.
- **Sensitivity:** The MitoPlate assays work with as little as 20,000 cells per well.
- **Broad Applicability:** The MitoPlates can be used with nearly any type of cell line or primary cell. The main requirement is that a uniform cell suspension must be prepared so that each well receives the same amount of cells. For these assays it does not matter if the cells are attached to the well bottom or floating in suspension.
- **High Resolution Analysis:** The Figure below highlights the numerous assay options including measurement of (1) Rates of electron flow from many NADH and $FADH_2$ producing mitochondrial substrates, (2) Sensitivity of electron flow to a) 22 diverse mitochondrial inhibitors using different mitochondrial substrates, b) novel drugs or chemicals, c) mutations in genes that alter mitochondrial function.

Mitochondrial Electron Flow Assay Options

NADH Producing Substrates	**Transporter Gene / Protein**	**Dehydrogenase Gene / Protein**
Pyruvate	MPC1 MPC2 / MPC	PDHA1 PDHA2 / Pyruvate DH
D-Isocitrate	SLC25A1 / CTP	IDH3A, IDH3B, IDH3G / Isocitrate DH
a-Ketoglutarate	SLC25A11 / OGC	OGDH / a-Ketoglutarate DH
L-Malate	SLC25A10, SLC25A1, SLC25A11 / DIC, CTP, OGC	MDH2 / Malate DH
Fatty Acids	SLC25A20 / CAC	HADH / 3-OH-Acyl-CoA DH

$FADH_2$ Producing Substrates	**Transporter Gene / Protein**	**Dehydrogenase Gene / Protein**
Succinate	SLC25A10, SLC25A1 / DIC, CTP	SDHA, SDHB, SDHC & SDHD / Complex II
Glycerol-3-P	None	GPD2 / G-3-P DH
Fatty Acids	SLC25A20 / CAC	SCAD, MCAD, LCAD, VLCAD & VLCAD2 / 5-Acyl-CoA DH



II. Product Descriptions

- **Products:** MitoPlates are 96-well microplates pre-coated with different tests which are dried on the bottom of each well. Other components of the assay are (1) A solution of the permeabilizing agent which must be prepared by the user, (2) The Biolog MAS solution which is osmotically optimized to preserve the physical structure of the cells following permeabilization, (3) The Biolog Redox Dye Mix which is used to measure the electron flow to the distal end of the electron transport chain, (4) A solution of the mitochondrial substrate which also must be prepared by the user. This solution is required only for the MitoPlate I-1.

- **Intended Use:** For Laboratory Use Only, to study the functional properties of mitochondria from permeabilized mammalian or other animal cells.

- **Product Storage:** MitoPlates should be refrigerated and stored at 4°C. Recommended storage conditions for chemical solutions are provided on their labels or should be determined by the user. MitoPlates may be taken out and prewarmed before use. For best results, use all products before the expiration date printed on the label.

- **Chemical Safety:** Safety Data Sheets for all products are available from Biolog and posted on the Biolog website at http://biolog.com/msds/.

III. Protocol Information

The protocol for MitoPlate S-1, the mitochondrial substrate plate, is provided on pages 5-6.

The protocol for MitoPlate I-1, the mitochondrial inhibitor plate, is provided on pages 7-9.

Preparation of Cells or Mitochondria

a. Cell Permeabilization

Whole cell assays require the use of permeabilizing agents that remove and sequester cholesterol from cell membranes. They work within seconds, allowing mitochondrial substrates and inhibitors to gain access to the mitochondria. We typically recommend a purified form of saponin, catalog number SAE0073 from Sigma-Aldrich. The effective range seems to be 12.5 to 100ug/ml, and generally speaking, 30 to 50ug/ml of saponin should be close to an optimal concentration for most cell types. Insufficient levels of saponin will give weak and partial permeabilization whereas excessive levels will damage the mitochondrial membrane, causing loss of mitochondria-associated electron flow and loss of sensitivity to mitochondrial inhibitors. Other permeabilizing agents may be substituted for saponin, for example digitonin or cholesterol-sequestering toxins, but these should be validated before use.

b. Mitochondria Purification

When it is not possible to use permeabilization, for example to assay mitochondria from tissue samples, mitochondria can be purified and assayed directly. Most purification methods require centrifugation, but a newer method gives excellent purification using filtration instead of centrifugation (J.M. Preble et al., (2014) J. Vis. Exp. (91) e51682).

c. Optimizing the Cell or Mitochondrial Concentration

30,000 cells per well or 50ug of mitochondrial protein per well is a good starting concentration. This may be adjusted up or down if the rate of color formation is too weak or too strong.

IV. MitoPlate S-1 Instructions For Use

MitoPlate S-1: Mitochondrial Function Assays Testing Substrates

A1 No Substrate	A2 α-D-Glucose	A3 Glycogen	A4 D-Glucose-1-PO4	A5 No Substrate	A6 α-D-Glucose	A7 Glycogen	A8 D-Glucose-1-PO4	A9 No Substrate	A10 α-D-Glucose	A11 Glycogen	A12 D-Glucose-1-PO4
B1 D-Glucose-6-PO4	B2 D-Gluconate-6-PO4	B3 D,L-α-Glycerol-PO4	B4 L-Lactic Acid	B5 D-Glucose-6-PO4	B6 D-Gluconate-6-PO4	B7 D,L-α-Glycerol-PO4	B8 L-Lactic Acid	B9 D-Glucose-6-PO4	B10 D-Gluconate-6-PO4	B11 D,L-α-Glycerol-PO4	B12 L-Lactic Acid
C1 Pyruvic Acid	C2 Citric Acid	C3 D,L-Isocitric Acid	C4 cis-Aconitic Acid	C5 Pyruvic Acid	C6 Citric Acid	C7 D,L-Isocitric Acid	C8 cis-Aconitic Acid	C9 Pyruvic Acid	C10 Citric Acid	C11 D,L-Isocitric Acid	C12 cis-Aconitic Acid
D1 α-Keto-Glutaric Acid	D2 Succinic Acid	D3 Fumaric Acid	D4 L-Malic Acid	D5 α-Keto-Glutaric Acid	D6 Succinic Acid	D7 Fumaric Acid	D8 L-Malic Acid	D9 α-Keto-Glutaric Acid	D10 Succinic Acid	D11 Fumaric Acid	D12 L-Malic Acid
E1 α-Keto-Butyric Acid	E2 D,L-β-Hydroxy-Butyric Acid	E3 L-Glutamic Acid	E4 L-Glutamine	E5 α-Keto-Butyric Acid	E6 D,L-β-Hydroxy-Butyric Acid	E7 L-Glutamic Acid	E8 L-Glutamine	E9 α-Keto-Butryric Acid	E10 D,L-β-Hydroxy-Butyric Acid	E11 L-Glutamic Acid	E12 L-Glutamine
F1 Ala-Gln	F2 L-Serine	F3 L-Ornithine	F4 Tryptamine	F5 Ala-Gln	F6 L-Serine	F7 L-Ornithine	F8 Tryptamine	F9 Ala-Gln	F10 L-Serine	F11 L-Ornithine	F12 Tryptamine
G1 L-Malic Acid 100uM	G2 Acetyl-L-Carnitine + L-Malic Acid 100uM	G3 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G4 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM	G5 L-Malic Acid 100uM	G6 Acetyl-L-Carnitine + L-Malic Acid 100uM	G7 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G8 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM	G9 L-Malic Acid 100uM	G10 Acetyl-L-Carnitine + L-Malic Acid 100uM	G11 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G12 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM
H1 Pyruvic Acid + L-Malic Acid 100uM	H2 γ-Amino-Butyric Acid + L-Malic Acid 100uM	H3 α-Keto-Isocaproic Acid + L-Malic Acid 100uM	H4 L-Leucine + L-Malic Acid 100uM	H5 Pyruvic Acid + L-Malic Acid 100uM	H6 γ-Amino-Butyric Acid + L-Malic Acid 100uM	H7 α-Keto-Isocaproic Acid + L-Malic Acid 100uM	H8 L-Leucine + L-Malic Acid 100uM	H9 Pyruvic Acid + L-Malic Acid 100uM	H10 γ-Amino-Butyric Acid + L-Malic Acid 100uM	H11 α-Keto-Isocaproic Acid + L-Malic Acid 100uM	H12 L-Leucine + L-Malic Acid 100uM

Intended Use: To assay the function of mitochondria from cells using mitochondrial substrates as probes.

MitoPlate Layout: The MitoPlate has a triplicate repeat of a set of 30 substrates (rows A-B cytoplasmic, rows C-H mitochondrial) precoated and dried into the wells. Either 3 assay samples can be run or one sample in triplicate. The mitochondrial substrates are transported via different transporters and metabolized using different dehydrogenases and electron transport chain components. The MitoPlate can also be used to assess the specificity of substrate transport inhibitors, dehydrogenase inhibitors, or electron transport chain inhibitors.

Assay Principle: Mitochondrial function can be assayed by measuring the rates of electron flow into and through the electron transport chain from metabolic substrates that produce NADH (e.g. L-malate, α-ketoglutarate, D-isocitrate, L-glutamate, D-β-hydroxy-butyrate) or $FADH_2$ (e.g. succinate, α-glycerol-PO4). Each substrate follows a different route using different transporters to enter the mitochondria, and then different dehydrogenases to produce NADH or $FADH_2$. The electrons travel from the beginning (complex 1 or 2) to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor that turns purple upon reduction.

Recommended Protocol:

Prepare in advance
2x Biolog MAS (Mitochondrial Assay Solution, Biolog cat# 72303)
6x Redox Dye MC (Biolog cat# 74353)
24x Saponin (e.g. 720ug/ml for 30ug/ml; 1200ug/ml for 50ug/ml; see III.a.)
Sterile water

Assay Mix:		Volumes per well	Volumes per plate	1.4x extra for pipetting
Combine	2x Biolog MAS	15ul	1500ul	2100ul
	6x Redox Dye MC	10ul	1000ul	1400ul
	24x Saponin	2.5ul	250ul	350ul
	Sterile water	2.5ul	250ul	350ul
Add to wells	TOTAL	30ul	3000ul	4200ul

Cell Suspension Preparation – 2x cells in 1x Biolog MAS

Harvest and resuspend cells in 1x Biolog MAS. Filter the cell suspension through a 70 micron nylon filter (cell strainer, Falcon 352350) to remove clumps. Count the cell number and determine their viability with trypan blue. The cells should have viability >95%.

Typical cell lines: For a final cell density of 30,000 cells per well, one plate requires a total of 3 x 10^6 cells in 3 ml of 1x Biolog MAS (1,000,000 cells per ml).

Typical blood cells: For a final cell density of 120,000 cells per well, one plate requires a total of 12 x 10^6 cells in 3 ml of 1x Biolog MAS (4,000,000 cells per ml).

Typical purified mitochondria: For a final concentration of 50ug of protein per well, one plate requires a total of 5mg of mitochondrial protein in 3 ml of 1x Biolog MAS. Saponin is not used and is replaced by sterile water.

Assay Steps:

1. Pipet 30ul per well of the Assay Mix into all wells and incubate at 37° C for 1 hour to allow substrates to fully dissolve.

2. Dispense the Cell Suspension to all wells by adding 30ul per well of the 2x cell suspension in 1x Biolog MAS.

3. Load the MitoPlate into the OmniLog® for kinetic reading of the rate of purple color formation. Alternatively, the color formation can be read kinetically on a microplate reader using OD_{590}.

Ordering Information:

Catalog #	Description
14105	MitoPlate S-1
72303	Biolog MAS
74353	Biolog Redox Dye Mix MC
96161	OmniLog PM-M System (NA Plug)
96162	OmniLog PM-M System (Schuko Plug)
96164	OmniLog PM-M System (UK Plug)
Not Included:	Saponin permeabilizing solution

V. MitoPlate I-1 Instructions For Use

MitoPlate I-1: Mitochondrial Function Assays Testing Inhibitors

A1 No inhibitor No substrate With Saponin	**A2** No inhibitor No substrate With Saponin	**A3** No inhibitor No substrate With Saponin	**A4** No inhibitor No substrate With Saponin	**A5** No inhibitor With substrate With Saponin	**A6** No inhibitor With substrate With Saponin	**A7** No inhibitor With substrate With Saponin	**A8** No inhibitor With substrate With Saponin	**A9** Meclizine 1	**A10** 2	**A11** 3	**A12** 4
B1 Complex I Inhibitor Rotenone 1	**B2** 2	**B3** 3	**B4** 4	**B5** Complex I Inhibitor Pyridaben 1	**B6** 2	**B7** 3	**B8** 4	**B9** Berberine 1	**B10** 2	**B11** 3	**B12** 4
C1 Complex II Inhibitor Malonate 1	**C2** 2	**C3** 3	**C4** 4	**C5** Complex II Inhibitor Carboxin 1	**C6** 2	**C7** 3	**C8** 4	**C9** Alexidine 1	**C10** 2	**C11** 3	**C12** 4
D1 Complex III Inhibitor Antimycin A 1	**D2** 2	**D3** 3	**D4** 4	**D5** Complex III Inhibitor Myxothiazol 1	**D6** 2	**D7** 3	**D8** 4	**D9** Phenformin 1	**D10** 2	**D11** 3	**D12** 4
E1 Uncoupler FCCP 1	**E2** 2	**E3** 3	**E4** 4	**E5** Uncoupler 2,4-Dinitrophenol 1	**E6** 2	**E7** 3	**E8** 4	**E9** Diclofenac 1	**E10** 2	**E11** 3	**E12** 4
F1 Ionophore, K Valinomycin 1	**F2** 2	**F3** 3	**F4** 4	**F5** Calcium $CaCl_2$ 1	**F6** 2	**F7** 3	**F8** 4	**F9** Celastrol 1	**F10** 2	**F11** 3	**F12** 4
G1 Gossypol 1	**G2** 2	**G3** 3	**G4** 4	**G5** Nordihydro- guaiaretic acid 1	**G6** 2	**G7** 3	**G8** 4	**G9** Trifluoperazine 1	**G10** 2	**G11** 3	**G12** 4
H1 Polymyxin B 1	**H2** 2	**H3** 3	**H4** 4	**H5** Amitriptyline 1	**H6** 2	**H7** 3	**H8** 4	**H9** Papaverine 1	**H10** 2	**H11** 3	**H12** 4

Intended Use: To assay the function of mitochondria from cells using mitochondrial inhibitors as probes.

MitoPlate Layout: The MitoPlate has 22 mitochondrial inhibitors at 4 dilutions precoated and dried into the wells. There are also 2 sets of control wells, each well repeated 4 times (negative control A1-A4 and positive control A5-A8).

Assay Principle: Mitochondrial function can be assayed by measuring the sensitivity of mitochondria to this set of 22 diverse inhibitors. The assays can be run with different metabolic substrates that produce NADH (e.g. L-malate, α-ketoglutarate, D-isocitrate, L-glutamate, D-β-hydroxy-butyrate) or $FADH_2$ (e.g. succinate, α-glycerol-PO4). Each substrate feeds electrons into the electron transport chain following a different route. The electrons travel from the beginning (complex 1 or 2) to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor that turns purple upon reduction. For example, a metabolic substrate that feeds complex 1 (L-malate) will result in a strong flow of electrons via malate dehydrogenase, which can be inhibited by either a complex 1 inhibitor (rotenone, pyridaben) or a complex 3 inhibitor (antimycin A, myxothiazol). A metabolic substrate that feeds complex 2 (succinate) will result in a strong flow of electrons via succinate dehydrogenase, which can be inhibited by either a complex 2 inhibitor (malonate, carboxin) or a complex 3 inhibitor (antimycin A, myxothiazol). The Reference section provides some references on the mode of action of the 22 inhibitors.

Recommended Protocol:

Prepare in advance: 2x Biolog MAS (Mitochondrial Assay Solution, Biolog cat# 72303)
6x Redox Dye MC, (Biolog cat# 74353)
24x Saponin (e.g. 720ug/ml for 30ug/ml; 1200ug/ml for 50ug/ml; see III.a.)
24x Substrate (e.g. 96mM sodium L-malate or succinate, pH7.2)
Sterile water

Assay Mix with Substrate:		Volumes per well	Volumes per plate	1.4x extra for pipetting
Combine	2x Biolog MAS	15ul	1500ul	2100ul
	6x Redox Dye MC	10ul	1000ul	1400ul
	24x Saponin	2.5ul	250ul	350ul
	24x Substrate	2.5ul	250ul	350ul
Add to wells	TOTAL	30ul	3000ul	4200ul

For negative control wells A1-A4

Assay Mix with No Substrate:		Volumes per well	Volumes per plate	4x extra for pipetting*
Combine	2x Biolog MAS	15ul	60ul	240ul
	6x Redox Dye MC	10ul	40ul	160ul
	24x Saponin	2.5ul	10ul	40ul
	Sterile water	2.5ul	10ul	40ul
Add to wells	TOTAL	30ul	120ul	480ul

* If using a multi-channel pipettor and a reagent reservoir, you will need 4x reagent volume to fill tips accurately. If you prefer to use a single-channel pipet for wells A1 – A4, you may use 1.4x volume.

Cell Suspension Preparation – 2x cells in 1x Biolog MAS

Harvest and resuspend cells in 1x Biolog MAS. Filter the cell suspension through a 70 micron nylon filter (cell strainer, Falcon 352350) to remove clumps. Count the cell number and determine their viability with trypan blue. The cells should have viability >95%.

Typical cell lines: For a final cell density of 30,000 cells per well, one plate requires a total of 3×10^6 cells in 3 ml of 1x Biolog MAS (1,000,000 cells per ml).

Typical blood cells: For a final cell density of 120,000 cells per well, one plate requires a total of 12×10^6 cells in 3 ml of 1x Biolog MAS (4,000,000 cells per ml).

Typical purified mitochondria: For a final concentration of 50ug of protein per well, one plate requires a total of 5mg of mitochondrial protein in 3 ml of 1x Biolog MAS. Saponin is not used and is replaced by sterile water.

Assay Steps:

1. Pipet 30ul per well of the Assay Mix with No Substrate into the negative control wells, A1-A4.

2. Pipet 30ul per well of the Assay Mix with Substrate into all other wells. Start with Column 12 and pipet from Column 12 to Column 5 using eight pipet tips. Then detach one pipet tip and fill wells B4-H4, B3-H3, B2-H2, and B1-H1.

3. Dispense the Cell Suspension to all wells by adding 30ul per well of the 2x cell suspension in 1x Biolog MAS.

4. Load the MitoPlate into the OmniLog® for kinetic reading of the rate of purple color formation. Alternatively, the color formation can be read kinetically on a microplate reader using OD_{590}.

Ordering Information:

Catalog #	Description
14104	MitoPlate I-1
72303	Biolog MAS
74353	Biolog Redox Dye Mix MC
96161	OmniLog PM-M System (NA Plug)
96162	OmniLog PM-M System (Schuko Plug)
96164	OmniLog PM-M System (UK Plug)
Not Included:	Saponin permeabilizing solution and substrate solutions for MitoPlate I-1

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VII. Frequently Asked Questions

What saponin product does Biolog recommend for permeabilization?

- From our experience, the best saponin product on the market that we have tested is Sigma Aldrich's saponin, product number SAE0073. It has the highest **sapogenin** content (20-35%) of any saponin product that we have tested, which we have correlated with having a broad window of efficacy.

What concentration of saponin do you recommend for my cell line?

- First we recommend that you run a preliminary test of your cell line in a MitoPlate S-1 using three concentrations of saponin and choosing the best concentration based on the highest signal from dye reduction. Of cells that we have tested, the optimal concentration has ranged between 10 and 100 µg/ml. Usually, the optimal concentration is about 50 µg/ml. Therefore we suggest testing 25-50-75 µg/ml or 30-50-70 µg/ml. However, it is always possible that the optimal concentration may be higher or lower for a specific type of cell or permeabilization may work better with digitonin instead of saponin.

My cells cannot be lifted and re-suspended. Can the assay still work for me?

- Yes, instead of adding the cell suspension to the MitoPlate, you can re-hydrate the 96 wells of the MitoPlate, and transfer the chemistry to another 96-well plate, containing the cells.

Can MitoPlates work for tissue samples?

- Yes, you can purify mitochondria from the tissue and use a mitochondrial suspension to inoculate the wells according to the protocol, leaving out the saponin permeabilization. When using this method, we recommend to standardize across tests by protein concentration, using approximately 50 to 100 µg of mitochondrial protein per well. Any good method of mitochondrial purification can be used, as long as organelle structure is maintained.

Why does it say that the cell suspension is at 2x?

- Because the cell suspension is added at 30 µl volumes to a well that already contains 30 µl of assay mix – the cell suspension becomes 1x once added to the well.

What volume does each plate require of each reagent?

- As described in the Instructions for Use (IFU) on page 6, calculations of final volumes are based on adding an extra 40% of each volume to provide some excess to allow for easier pipetting. With this in mind, use about 4.6 ml of Biolog MAS Buffer (this includes the volume needed for the cell suspension), and 1.4 ml of Dye Mix MC per plate.

How do I read the MitoPlates?

- The best option is to use an OmniLog® PM-M System which is a multiplate kinetic reader with temperature control. In addition, the plates can also be read on a microplate reader. In this case, we recommend to read OD at both 590 nm and 750 nm, and then subtract signal at 750 nm from 590 nm, at every read point. This is to minimize any abiotic background. A typical assay should run for 2 to 6 hours with 5 minute read intervals, if possible.

Should the MitoPlates be read with the lid on or lid off?

- Fogging of the lid or scratches and other non-uniformities will scatter the light beam and cause false readings. Therefore the lids should be removed when reading the plates.

At what temperature should I read the MitoPlates?

- Plates should be kept at 35-37° C if possible for mammalian cells.

How do I analyze the data from reading MitoPlates?

- In our experience, the best data is the initial linear slope rate in the first 2 hours.

How can I increase the signal & reproducibility, and decrease the background & noise?

- The signal can be increased by optimizing the saponin concentration and/or adding more cells per well. Reproducibility is obtained by careful and gentle handling and pipetting of all solutions, especially the cell suspension. A common source of background and noise is the formation of bubbles which can occur due to foaming by the saponin and by too rapid filling and dispensing with the pipetter. Better results can be obtained by slow and careful filling and dispensing. Bubbles will cause light scattering and noise when measuring absorbance in a microplate reader.