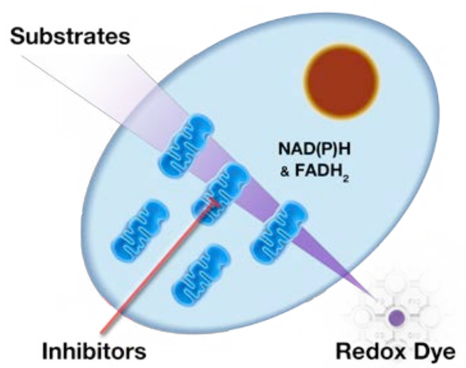
MitoPlate™ S-1 and MitoPlate™ I-1

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

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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

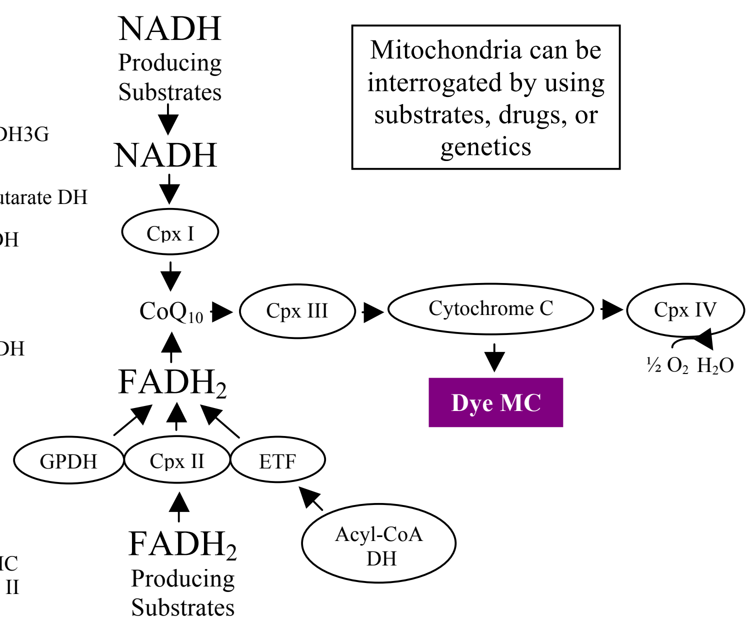
MitoPlates provide a unique and powerful tool for simultaneously running 96 relevant assays to elucidate changes in mitochondrial properties. Some principal advantages are:

- **Proven Technology:** A sizeable published literature documents the successful use of Biolog's PMM 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^{o}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.

a. Cell Number Optimization

Increasing the number of cells per well will increase the rate of dye reduction. Generally speaking, 30,000 cells per well should be close to ideal. This may be adjusted up or down if the color formation is too weak or too strong.

b. Cell Permeabilization Optimization

The cell permeabilization concentration can also be adjusted for different types of cells. Generally speaking, 30ug/ml of saponin should be close to an ideal concentration. Increasing the concentration will usually increase the rate of color formation, but it may also 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 must be validated before use. It is possible that the activity of these permeabilizing agents may change depending on the vendor and the production lot.

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.

MicroPlate Layout: The MicroPlate has a triplicate repeat of a set of 31 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 MicroPlate 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; 2400ug/ml for 100ug/ml)
	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%.

For a final cell density of 20,000 cells per well, one plate requires a total of 2 x 10^6 cells in 3 ml of 1x Biolog MAS (666,667 cells per ml).

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).

For a final cell density of 40,000 cells per well, one plate requires a total of 4 x 10^6 cells in 3 ml of 1x Biolog MAS (1,333,334 cells per ml).

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 MicroPlate 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
74354	Biolog Redox Dye Mix MD
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.

MicroPlate Layout: The MicroPlate 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 (3-nitropropionic acid, 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; 2400ug/ml for 100ug/ml)
- 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%.

For a final cell density of 20,000 cells per well, one plate requires a total of 2 x 10^6 cells in 3 ml of 1x Biolog MAS (666,667 cells per ml).

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).

For a final cell density of 40,000 cells per well, one plate requires a total of 4 x 10^6 cells in 3 ml of 1x Biolog MAS (1,333,334 cells per ml).

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 MicroPlate 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
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74354	Biolog Redox Dye Mix MD
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

VI. References

Meclizine

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