New Mitochondrial Function Assay Technology

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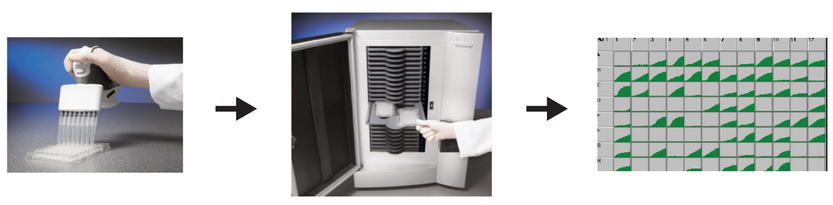
Abstract

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 $FADH_2$.

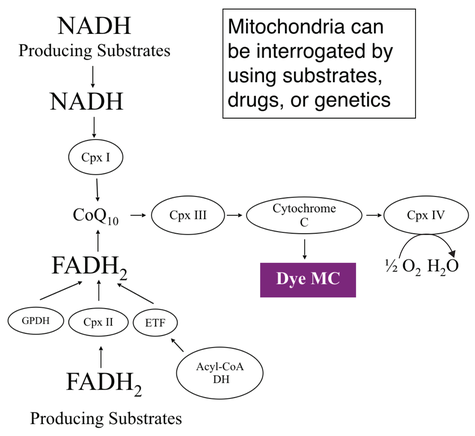


Figure 3. The MitoPlate S-1 simultaneously assays the metabolic rates of potential NADH or $FADH_2$ producing substrates.

MitoPlate S-1: Mitochondrial Function Assays Testing Substrates

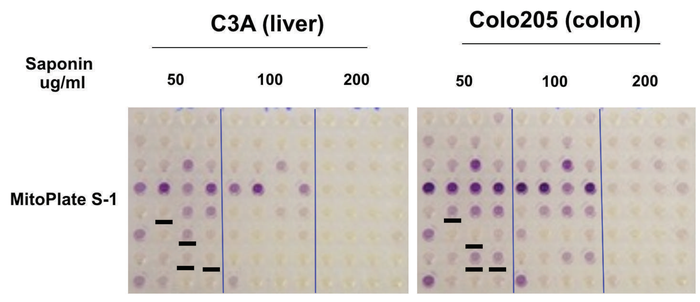
A1 No Substrate	A2 α-D-Glucose	A3 Glycogen	A4 D-Glucose-1-PO4	A5 No Substrate	A6 α-D-Glucose	A7 Glycogen	A8 D-Glucose-1-PO4	A9 No Substrate	A10 α-D-Glucose	A11 Glycogen	A12 D-Glucose-1-PO4
B1 D-Glucose-6-PO4	B2 D-Gluconate-6-PO4	B3 D,L-α-Glycerol-PO4	B4 L-Lactic Acid	B5 D-Glucose-6-PO4	B6 D-Gluconate-6-PO4	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.

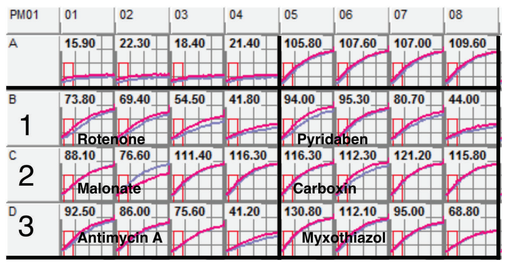
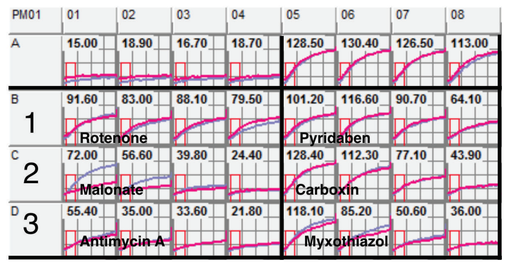


Figure 7. Colon cells were assayed in MitoPlate I-1 with succinate as the substrate. The cells were sensitive to Complex 2 and 3 inhibitors, but not Complex 1 inhibitors.



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.