MITOCOHDRIAL
TOXICITY SCREENING
APPLICATION GUIDE

MitoSciences
Advancing vital discoveries in mitochondrial research
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I. Why is Testing for Mitochondrial Toxicity Important?

Mitochondria perform two critical functions in the cell, namely the production of more than 90% of the cell's energy, and the control of cell survival as an integral part of programmed cell death (apoptosis). Unwanted changes in either of these functions can have dire consequences, and hence monitoring compounds for mitochondrial toxicity is a crucial element of any drug toxicity screening program, and of many investigational toxicology studies as well.

Every year an appreciable percentage of drugs are withdrawn from the market, or their use is curtailed by black box warnings, due to some adverse effect that was not discovered during preclinical and clinical testing. This is often because the adverse effect is subtle and does not lead to histopathology.

Recently, and with a better understanding of mitochondrial structure and function, newer assays for mitochondrial dysfunction have been developed. Application of these assays has revealed strong mitochondrial impairment by several previously withdrawn drugs including cerivastatin (Baycol), troglitazone (Rezulin), nefazodone (Serzone), and tolcapone. Not surprisingly, there is now much more focus on identifying mitochondrial toxicity early in the development process.

II. What Testing Options Are Available?

There are three general adverse effects that result from mitochondrial toxicity: 1. disrupted energy metabolism; 2. increased free radical generation; and 3. altered apoptosis.

The ability to test drugs for these effects is advancing rapidly. For several years toxicity panels have been available for cell-based screening systems such as Cellomics®, and these panels typically include one or more mitochondrial toxicity assays, such as assays for ATP production, membrane potential, caspase 3 activation (apoptosis), and reactive oxygen species. These assays present a useful first-order screen for general mitochondrial health, but none of these assays can, even in combination, provide the mechanistic data on which yes/no decisions can confidently be made about the safety of a drug, nor can they guide compound redesign efforts. These assays are also best-suited to identifying acute toxic effects, and chronic effects on mitochondrial protein expression and post-translational modifications may be missed.

The MitoSciences set of assays extends the data-gathering ability of the toxicologist by providing a set of investigational assays that allow for the identification and measurement of the specific sites and mechanisms of toxicity at the individual protein level.
The MitoSciences assays are able to measure the activity and expression of key metabolic enzymes, plus their phosphorylation and acetylation, to elucidate effects on energy metabolism. They can measure protein-specific nitration and carbonylation to elucidate effects from oxidative stress, and they can measure changes in expression and translocation within the cell of key pro- and anti-apoptotic proteins.

<table>
<thead>
<tr>
<th>Toxic Effect</th>
<th>Energy Metabolism Disruption</th>
<th>Free Radical Production</th>
<th>Apoptosis Induction</th>
</tr>
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<tbody>
<tr>
<td>Screening Assays</td>
<td>ATP Production</td>
<td>ROS Production</td>
<td>Caspase 3 Activation</td>
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<td></td>
<td>Membrane Potential</td>
<td>Glutathione Expression</td>
<td>PARP Activation</td>
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<td></td>
<td>Oxygen Utilization</td>
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<td></td>
<td>mt Biogenesis</td>
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<tr>
<td>Mechanistic Assays</td>
<td>OXPHOS Enzyme Activity</td>
<td>Protein Nitration Panel</td>
<td>Cell Fractionation &amp; Protein Translocation Panel</td>
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<td></td>
<td>Phospho. &amp; Acetyl.</td>
<td>Protein Oxidation Panel</td>
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<td></td>
<td>Metabolic Protein Expression Panel</td>
<td>Antioxidant Protein Expression Panel</td>
<td>Apoptosis Protein Expression Panel</td>
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</tbody>
</table>

**Figure 1.** Mitochondrial toxicity testing paradigm. The three general toxic effects can be measured using assays suitable for both screening as well as those suited for detailed mechanism-of-action studies. All of the assays in the paradigm can be performed in 96-well plates. All of the screening assays other than the mitochondrial biogenesis assay are available from a variety of vendors, while the biogenesis assay plus the rest of the mechanistic assays are proprietary to Mitosciences.
Stage 1: Screening for General Mitochondrial Function
A typical mitochondrial toxicity testing project begins with cell-based screening assays, all of which can be run in high-throughput, and, with the exception of the mitochondrial biogenesis assay, all of which measure acute toxicity parameters and are available from a variety of vendors. The biogenesis assay is proprietary to MitoSciences, and it is a chronic tox test recommended at the screening stage particularly for antibiotic and anti-viral drugs, for reasons described below.

Stage 2: Investigating Mechanism of Action
The second step in a mitochondrial tox study is the deployment of a set of MitoSciences assays to generate further detail on the site(s) and mechanism(s) of action of any toxicity discovered during the screening stage, or to generate data to ensure that all possible chronic effects of a drug on mitochondrial function have been ruled-out. The MitoSciences assays can be run in high-throughput for the efficient generation of IC_{50}'s, and many of them are also available in dipstick form for rapid and simple testing of animal or patient samples.

Both Kit and Service Offerings Available
MitoSciences offers all of our assays in kit form, and we can provide extensive support in helping a customer get the assays performing optimally in their own labs or at their CRO. MitoSciences can also direct the customer to the best suppliers for the Stage 1 screening assays, and indeed will soon offer them in bundled form. For clients who prefer to outsource the performance of either the Stage 1 or Stage 2 assays, MitoSciences offers the MitoTox™ contract research service, in which complete support is provided, from the experimental design, through the performance of the assays, and ultimately to the final data analysis.

The MitoTox™ Database
For customers who commit to supply agreements for kits, or who contract MitoSciences for the MitoTox™ service offering, an extensive data set is available covering a wide range of drugs from multiple drug classes, all of which have been run through both the Stage 1 and Stage 2 assays in a variety of cell lines. This database is freely shared with our customers to provide the basis for a comprehensive comparative analysis.
III. Stage 1: Screening for General Mitochondrial Function

The assays used to conduct initial screening are typically performed on cell cultures within 6-24 hours of drug-dosing to identify acute effects.

The assays in this panel include:

A. ATP Production
Reduction in ATP production can be a primary event caused by a direct effect on mitochondria, or a secondary event due to altered metabolism through any of several interactions of the compound within the cell.

B. Mitochondrial Membrane Potential
Membrane potential measurement provides information on the coupling ability of electron transfer to ATP synthesis, as well as the ability of the organelle to take up and release ions and substrates across the mitochondrial inner membrane.

C. Oxygen Consumption
Measurement of oxygen consumption complements assays of ATP and membrane potential, as O$_2$ utilization by the cell is a function of both throughput of the electron transport chain and coupling of this process to ATP production. Moreover, the rate of O$_2$ consumption is linked directly to the membrane potential because uncoupling, and the resultant loss of proton translocation across the mitochondrial inner membrane, causes a loss of membrane potential. A determination of O$_2$ consumption is the best way to identify compounds that act as uncouplers, and these are often, but not always, weak hydrophobic acids.

D. ROS Production & Total Glutathione
Increased free radical production is a feature of mitochondrial dysfunction that can be measured both by dye-based assays of superoxide generation as well as changes in reduced glutathione levels. The predominant source of free radical generation is the mitochondrial respiratory chain, and inhibition of this process is often connected to increased levels of free radicals. However there are other cellular sources of free radicals in cells, and so it is not an unequivocal indicator of a direct effect of the compound on the organelle. Also, the standard assay of free radical scavengers does not measure the chronic consequences of increased free radical generation, hence the need for measurement of oxidative and nitrative enzyme modifications, and of modification-induced loss of enzyme activities.

E. Caspase 3 & PARP Activation
Induction of apoptosis is a critical measurement to make, and several different parameters of this process are often determined in high-content screens, including changes in the plasma membrane distribution of phosphatidylserine and activation of caspases.
Mitochondrial dysfunction can induce apoptosis and there is a link between loss of ATP production and apoptosis that appears to be correlated to free radical production. Apoptosis can also be triggered by many cellular events that do not involve mitochondrial dysfunction directly, including chromosomal damage, but inevitably the organelle becomes involved as the central executioner.

F. Mitochondrial Biogenesis
A compound that does not exhibit toxic effects in the aforementioned screening assays, even at high concentrations, cannot be considered free of mitochondrial toxicity until the potential for chronic toxicity has been evaluated. This requires culturing cells with the compound for as long as a week, or until there have been several cell divisions. This is particularly important for antibacterials and antivirals, both of which can affect mitochondrial biogenesis.

**Figure 3A** shows maximal inhibition of mtBiogenesis after multiple cell divisions by a number of different anti-bacterials and anti-virals, while **Figure 3B** shows the loss of mitochondrial-encoded proteins induced by the antibiotic chloramphenicol, as a function of dosage. The mitochondrial biogenesis assay is available in high throughput 96-well format (MS641) useful for cell-based experiments and animal studies, and on a dipstick assay platform (MS631) useful for animal studies and clinical trials.

Mitochondria are unique among animal cell organelles in having their own DNA and their own protein synthesis apparatus. Both features are evolutionary remnants from the early symbiotic fusion of bacterial cells to produce eukaryotes. For drug screening this has important consequences, as the mitochondrial replication and translational machinery are more similar to their bacterial counterparts than to the cellular cytosolic/nuclear corresponding systems. Thus any evaluation of the toxicity of anti-bacterial or antiviral drugs designed to attack bacterial polymerases or their protein synthesis machinery must be evaluated for their effect on mitochondria.
The Mitochondrial Biogenesis assay quantitatively measures the levels of an enzyme whose presence depends on mitochondrial replication and protein synthesis (cytochrome c oxidase) with that of a mitochondrial protein that is encoded on the nuclear DNA and translated in the cytosol (frataxin).

**Guiding Stage 2 Studies**

The findings of a Stage 1 screen provide an important guide as to which of the MitoSciences assays should be performed in Stage 2.

Whether there is altered **energy metabolism** or not based on ATP production, change in oxygen utilization, and/or change in membrane potential, the actual mechanism of action behind such changes can only be revealed by examining effects at the protein level. General screens for **oxidative stress** can be even less revealing, as early compensatory events such as up-regulation of antioxidant proteins can mask a toxic event that can only be identified by detailed proteomic analysis. Early screens for **apoptosis** provide greater confidence, and so a compound that does not induce apoptosis in Stage 1 screening generally only requires more detailed analysis if the lack of apoptosis was unexpected.
IV. Stage 2: Investigating Mechanism of Action

Each of the MitoSciences assays described below is available in kit form. All assays are easy to perform and can be carried out in high throughput, or as needed on limited numbers of samples in a cost effective way.

A. OXPHOS Enzyme Activity Assays
*For measuring the direct effect of drugs on the key respiratory chain enzymes.*

Many drugs are inhibitors of one or more of these complexes, and such an interaction can be a primary cause of cell toxicity if the compound can enter mitochondria, which many do. In fact, a significant portion of compounds, and particularly those weak, hydrophobic acids, can become concentrated in mitochondria.

<table>
<thead>
<tr>
<th>Compound</th>
<th>OX1</th>
<th>OX2+3</th>
<th>OX4</th>
<th>OX5</th>
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<tbody>
<tr>
<td>Antimycin</td>
<td>101%</td>
<td>11%</td>
<td>62%</td>
<td>75%</td>
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<tr>
<td>BILN 2061</td>
<td>115%</td>
<td>47%</td>
<td>53%</td>
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<td>Chlorpromazine</td>
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<td>76%</td>
<td>118%</td>
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<td>Ciglitazone</td>
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<td>52%</td>
<td>77%</td>
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<tr>
<td>Fenofibrate</td>
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<td>89%</td>
<td>105%</td>
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<td>Fluoxetine</td>
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<td>86%</td>
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<tr>
<td>Iressa</td>
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<td>82%</td>
<td>85%</td>
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<td>KCN</td>
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<td>96%</td>
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<tr>
<td>Lopinavir</td>
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<td>61%</td>
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<td>90%</td>
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<tr>
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<td>33%</td>
</tr>
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<td>TTFA</td>
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<tr>
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<td>68%</td>
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**Table 1.** Selection of compounds with direct inhibitory effects on the respiratory chain complexes. Data is shown as the average of triplicate measurements and it is normalized as percentage from DMSO control. OX1 measures the activity of Complex I, OX2+3 measures the activity of Complexes II&III, OX4 measures the activity of Complex IV, and OX5 measures the activity of ATP synthase.

**Figure 4.** Dose response effect of ATP synthase (Complex V) and succinate ubiquinone oxidoreductase (Complex II) activity in the presence of Troglitazone and Simvastatin respectively. Activity is shown on the X axis and it is given as percentage from DMSO control. Concentration of compounds is shown on the Y axis and is given in the micromolar range.
B. Cell Fractionation & Apoptotic Protein Translocation Assays

*For measuring protein movements between cell compartments as a result of a drug treatment.*

The relocation of proteins is an important part of metabolic cell signaling and it is a key irreversible step in apoptosis. The release of cytochrome c and other apoptotic factors from mitochondria is the point of no return in the apoptotic process. In addition, there is emerging evidence of migration of transcription factors between mitochondrial and other cellular compartments. Such movements are key steps in the overall control of mitochondrial biogenesis and function as well as in the execution of apoptosis.

![Figure 5. Protein movement into and out of mitochondrion in apoptosis.](image)

Figure 5 shows some of the key movements of apoptotic factors during programmed cell death. Monitoring of these provides insight into the nature and site of apoptotic signaling, and hence the mode of action of a compound on the apoptotic machinery.
The key to the MitoSciences set of tools for monitoring apoptosis and other physiologically relevant movements of proteins between cellular organelles is development of a rapid and simple approach to separate mitochondrial, cytosolic and nuclear fractions that does not require mechanical disruption. This protocol yields essentially full separation of the three compartments, allowing for quantitative assessment of protein movements as a result of any drug action. Figure 6 shows the distribution achieved when HepG2 cells are fractionated with our protocol and then probed with a set of Western blotting cocktails or by sandwich ELISA for determination of protein amount.

When cells undergoing apoptosis are examined in the same way, any redistribution of proteins can be detected and quantified. Figure 6 shows the example of apoptosis induced by Gleevec in cardiomyocytes. In addition to release of cytochrome c and SMAC Diablo (not shown for simplicity), there is a sizable relocation of Bax, activation (cleavage) and redistribution of PARP and movement of GAPDH from a soluble form in the cytosol.
With different apoptotic stimuli, involvement of, and movements of, Bid, p53, PUMA etc can be followed using appropriate mAb cocktails.

C. Protein Expression and Post-Translational Modification Assays

For measuring metabolic, antioxidant, and pro-anti-apoptotic protein levels, plus, wherever relevant, their phosphorylation, acetylation & oxidative/nitrative modifications.

Cells contain acute and long term mechanisms of adaptation to drug-induced cellular stress. Acute changes are based on the regulation of metabolism through post-translational modifications such as phosphorylation/dephosphorylation and acetylation/deactylation events. Long term adaptation is based on changes in the levels of protein expression.

MitoSciences provides several tools for analyzing such stress-induced changes. First we provide our “global” protein expression screen, the MetabArray™ chip, which is an antibody microarray that captures 50+ key metabolic, antioxidant, and apoptotic protein targets that were selected based on their changing as a result of drug treatments, or based on literature reports of their up- or down-regulation during cellular stress.

When cells are compared before and after drug-treatment, changes in the expression of a broad range of enzymes can thus be analyzed qualitatively to identify the various metabolic responses induced. New mAbs are being added to this array regularly.

Figure 8. Quantitation of nitrotyrosine modification, represented as signal to background, after mitochondria are exposed in vitro to peroxynitrite (800 µM). Many enzymes are modified presumably as a function of surface-accessible tyrosine residues.
Along with the protein expression measurement, the MetAbArray™ can also be duplexed with measurements of phosphorylation and acetylation. Recent studies identify changes in the levels of both modifications, directed by the many cell stress signaling pathways as an important determinant of the response to cell perturbations, and so it is not surprising that such changes provide a fingerprint of drug effects.

In addition to duplexing with these modifications, the MetAbArray™ can also be duplexed with measurements of oxidative damage. The array can thus provide insight into oxidative stress by measuring not only the up- or down-regulation of key free radical scavengers such as SOD1, SOD2 and catalase, but it can also identify the extent to which any acute oxidative event has overcome normal protective processes by measuring oxidative modifications of key metabolic enzymes. Figure 8 shows the susceptibility of selected enzymes to nitrotyrosine modification when cells are exposed to peroxynitrite.

An important aspect of the MitoSciences capture mAbs is that they are all screened for their ability to capture native and ACTIVE enzymes. Therefore, as studies zero-in on specific proteins as potential biomarkers for a particular compound action or unwanted toxicity, the effects on these activities can be obtained as a specific activity (change).

(A) Oxidative stress and effect on activity

![Graph showing activity and 3-nitrotyrosine labeling](image)

_B Figure 9._ The effects of oxidative stress on the enzyme pyruvate dehydrogenase (PDH), the key regulator of oxidative metabolism, is studied in greater detail.

(A) As nitrination of tyrosines increases with exposure to oxidizing conditions, activity decreases due to site-specific modifications.

(B) After purification using immunocapture beads the site of modification is clearly the E2 subunit which contains a functionally necessary lipoyl domain. Lipoic acid is a well-established anti-oxidant.
An example is shown in figure 9. In this study the specific activity of the pyruvate dehydrogenase complex (PDH) was measured after treating cells to induce oxidative stress.

Once an overview of the effects of a drug has been obtained, more specific and highly quantitative changes in the levels and post-translational modifications of sets of enzymes can be monitored using 4-plex MetaPath™ arrays, or individual enzymes can be monitored with singleplex MitoProfile® assays. Both assay families are available in both microplate and dipstick formats.

MetaPath™ and MitoProfile® assays are all sandwich ELISA’s, in which capture/detector antibody pairs are used to provide a highly quantitative measurement of protein expression or post-translational modification.

MetaPath™ arrays are 4-plex assays in which sets of analytes are grouped according to metabolic pathway or by their experimental relevance for a given research question. In the high-throughput format of these arrays, capture antibodies are spotted in 96-well plates, and in the rapid and simple format they are striped onto dipsticks. Protein expression or PTM’s are measured using detector antibody mixes. Microplate arrays are ideal for screening applications while dipsticks are ideal for testing small sample volumes gathered from animal or patient.

![Image of 4-plex MetaPath™ Microplate Array]

**Figure 10.** Schematic of the 4-plex MetaPath™ Microplate Array. The array can be read by a variety of imaging systems.

MitoProfile® assays use the same concept but measure only one analyte per plate or dipstick, allowing for greater efficiency when the importance of the target protein has been well-established and multiplexed measurements are no longer required.
V. Important Questions to Consider When Conducting Cell-Based Mitochondrial Toxicity Studies

There are several experimental issues to address before beginning any major screen of compounds for their mitochondrial toxicity. These involve: cell type to be used, growth medium for the cells, serum concentrations in the media, concentration of compound, and time/length of incubation before any mitochondrial parameters can be measured.

A. Which Cell Line(s) Should I Use?
At first, choosing a cell line seems like a relatively easy decision. A priori, it makes sense to use a cell line derived from the tissue or tissues that you believe are most likely to be positively/negatively affected by the compound class. However this is not a straightforward choice. First, the process of generating cells from a tissue source can select a sub-class of cells that are not representative of the entire tissue. Second, both primary and transformed cell lines by virtue of their growth in culture have accommodated to in vitro growth conditions, which dramatically differ from the in vivo environment. It is standard practice to grow cells in a glucose medium. When cells are provided a medium rich in glucose they become lazy and reduce their dependence on OXPHOS in favor of generating most of the ATP needs by glycolysis. As a result, the effects of a compound on energy metabolism, particularly on oxidative phosphorylation, can be underestimated.

Furthermore a switch on metabolic substrate generates changes in expression of many proteins, not just the ones directly involved in OXPHOS or lipid oxidation. The end result is that the cell line is not fully representative of the tissue from which it was derived, particularly in terms of mitochondrial function. Figure 11 shows protein expression changes between heart tissue and its derived cardiomyocytes cultured in standard growth media. Similar broad differences have been reported between the protein expression pattern of liver tissue and primary hepatocytes.

Another important issue for primary cell lines when it comes to evaluating the mitochondrial effect of a compound is that there are often limits to the number of divisions that the cells can go through before senescence or death. This is problematic when studying induced mitochondrial dysfunction because of the unique character of the organelle; it has its own DNA. The number of copies of this DNA is on the order of 1000s per cell and therefore the effect of compounds affecting mtDNA replication can take several cell passages before the threshold for viable mitochondrial protein production is surpassed and the pathogenic effect observed. These effects are often missed when primary cell lines are chosen. The alternative to primary cells is to use transformed cell lines e.g. HepG2 cells (which are very commonly used). Such cells can be kept in culture for many cell divisions. However, it is important to consider that their transformation has reprogrammed energy metabolism permanently. This is the so-called Warburg effect.
Figure 11. A comparison of the expression of key metabolic and apoptotic enzymes in human cardiomyocytes with the cardiac tissue from which these cells were derived. These samples have similarities including expression of several enzymes where the expression ratio is close to 1. Additionally these tissues share a lack of expression of several key enzymes of metabolism including Acetyl coA carboxylase, fatty acid synthase, lactate dehydrogenase and cytoplasmic antioxidant enzymes superoxide dismutase 1 and thioredoxin 1. However, the cardiomyocytes also show significant differences in protein expression from their parent tissues including reduced expression of glycogen synthase and increased expression of multiple pro-apoptotic proteins including Bad, Bax, Bid, caspase-3, PUMA. The number of proteins whose expression has changed is very high and many of these are involved in apoptosis as well as in other mitochondrial functions.

Figure 12 compares the protein composition of several of the cell lines commonly used in toxicity studies. Note that there are significant differences in the relative amounts not only of the proteins involved in energy metabolism, as discussed above, but also of several proteins that control apoptosis.
**Figure 12.** The expression profile of metabolic and apoptotic enzymes differs between cells commonly used for toxicity studies and is dynamic. For this example the expression of proteins in four cell lines is related to a commonly used transformed liver cell line (HepG2). A decrease in expression below 25% is represented in blue, while increased expression greater than 200% is represented in red. Metabolic expression differences occur between this and other transformed cells adenocarcinoma (column 1) and lymphoblast cells (column 2). In the a primary cell line, fibroblasts (column 3), several proteins are significantly decreased while the expression of several proteins are up regulated by simply switching the carbon source of the cell line from glucose to galactose promoting mitochondrial oxidative metabolism (column 4).

**B. What Cell Culture Conditions Should I Use?**
This is not a trivial issue. Cells in culture, whether primary or transformed, prefer to use glycolysis rather than oxidative phosphorylation to produce ATP when grown in glucose. This is potentially problematic when looking for adverse effects of a compound on energy metabolism, particularly if effects on OXPHOS are being assessed. One way around the problem is to use galactose plus glutamine as a substrate. The arguments for using this combination and the way that the cell is fed with reducing equivalents for oxidative phosphorylation are described in two recent papers and will not be reviewed here [9, 10].
Another important issue when cells are grown in high glucose, as many researchers do, is the high levels of free radicals they produce and the significant oxidative modification of key metabolic enzymes they show. We recommend screening compounds initially in both high glucose and in galactose plus glutamine. In the absence of a dual screen, we recommend to choose the galactose buffer conditions with compounds suspected to have an effect on energy metabolism.

The levels of serum in the media are also an important variable in the experimental design. Many compounds (such as Troglitazone) are known to bind to serum proteins. Acute treatments of Troglitazone in low serum media will generate dramatic changes in the levels of many mitochondrial proteins that are not observed in the presence of high serum media. Serum deprivation can activate apoptosis signaling in many cell lines which could overestimate toxicity, and so the level of serum must be adjusted in any experimental design according to the specific questions that the researcher wants to ask about the drugs being screened.

C. How Long Should I Culture My Drug in Cells Before Making Metabolic Measurements?

As mentioned above, compounds can have an acute effect on cellular metabolism due to direct enzyme inhibition, altered cell signaling and post-translational modifications. Some toxic events occur rapidly and the cells die soon after, so that long term effects such as apoptosis must be measured relatively early. Many compounds induce long term effects, often changes in protein expression. When the levels of a protein are reduced by inhibition of gene expression, the ideal timing for capturing the event depends on the half life of the proteins involved which varies from one protein to another. If a drug affects mitochondrial DNA replication the effect is only evident after several cell divisions. Free radical generation can occur early but disappear later, presumably by compensation through increased production of anti-oxidant proteins. In short, the length of time of treatment with a compound depends on the parameters to be measured. We generally begin by monitoring cellular events after incubating with a compound for 6, 24 and 72 hrs. When we are dealing with compounds thought to affect mitochondrial biogenesis we set up cultures for incubation with the drug for 7 days.
VI. Useful Reviews & Publications


VII. Selected Assays for Drug Toxicity Studies

- **MS641 MitoBiogenesis™ Microplate Assay Kit**
  A 96-well plate assay for measuring effects on mtDNA replication and mitochondrial protein synthesis. Also available as a dipstick assay (MS631) for rapid and simple testing of animal or patient samples.

- **MT-OXC Complete OXPHOS Enzyme Activity Microplate Assay Panel**
  A set of (5) 96-well plate assays for measuring the direct effect of drugs on the activity of the 5 OXPHOS enzyme complexes.

- **MSM1 MetAbArray™ Total Protein Microarray**
  A slide array for qualitatively measuring changes in the expression of 50+ key metabolic, antioxidant, and apoptotic proteins. Can be duplexed with measurements of tyrosine phosphorylation, acetylation, nitration, or carbonylation.

- **MSX41 MetaPath™ OXPHOS+PDH Total Protein Microplate Array**
  A 4-plex array for quantitatively measuring the expression of Complex I, Complex IV and PDH, plus a reference protein (frataxin). Also used for measuring effects on mitochondrial biogenesis. Also available as a dipstick array (MSX31) for rapid and simple testing of animal or patient samples.

- **MSX42 MetaPath™ Fatty Acid Total Protein Microplate Array**
  A 4-plex array for quantitatively measuring the expression of 3 key enzymes of Fatty Acid Oxidation - MCAD, SCHAD and TFP, plus a reference protein (frataxin). Also available as a dipstick array (MSX32) for rapid and simple testing of animal or patient samples.

- **MS862 Cell Fractionation Kit HT**
  A complete kit for isolating in high-throughput a mitochondrial, cytosolic and nuclear fraction from cells grown in 96-well plates without the need for mechanical disruption. Ideal for use with ELISA assays or WB cocktails in Stage 2 studies to follow the translocation of proteins during apoptosis.

- **MSA12 ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail**
  Allows for the detection of cytochrome c in cytoplasmic and mitochondria-containing fractions for determining the proportion of released cytochrome c from mitochondria to the cytoplasm from apoptosis. The kit also includes antibodies against a cytoplasmic protein (GAPDH), and 2 mitochondrial markers (PDH, a matrix marker, and ATP synthase, an inner membrane marker). This set of control markers allows for the monitoring and/or optimization of the permeabilization conditions. Ideal for confirming the results of an early high-throughput screen for cytochrome c release.

- **MSA41 Cytochrome c Protein Quantity Microplate Assay Kit**
  A 96-well microplate immunoassay that is used to determine the amount of cytochrome c in a human, mouse, rat or bovine sample.

In addition to the above assays, MitoSciences also offers numerous microplate and dipstick assays against individual proteins for a more comprehensive analysis of any single target.

Please see a complete list at http://www.mitosciences.com
VIII. The MitoTox™ Service

For customers that prefer to out-source their mitochondrial toxicity screening, or who require assistance in establishing an in-house program, MitoSciences offers the MitoTox™ service.

A. Screening Solutions
Having developed many of the assays deployed in mitochondrial screening, and having extensively used all of the available technologies for conducting such studies, MitoSciences is well-positioned to design your experiments, perform them in our labs, and assist with the analysis of data.

Stage 1
Assay Set: Standard Cell Lines*:
1. Total ATP 1. HepG2
2. Mitochondrial Membrane Potential 2. HeLa
3. ROS & Total Glutathione 3. SHSY5Y
4. Caspase 3 & PARP Activation

Pricing

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*Other cell lines are available but may include additional set up and culturing costs.

Stage 2
The set of assays conducted in Stage 2 studies is defined by the findings in Stage 1, and your MitoSciences Study Director will work closely with the rest our science team to generate the most efficient next step for achieving your study goals.

B. Consulting Solutions
MitoSciences has helped to set up mitochondrial toxicity screening programs at multiple drug companies both large and small, and we can assist with every aspect of such an effort. We help you to identify the assays and cell lines to establish, we can train your team on the performance of the assays, and we can help you to incorporate data from the MitoTox™ database into your existing bioinformatics system.