

# Screening for Mitochondrial Toxicity

A protocol for the isolation of mitochondria and measurement using the Agilent MitoXpress Xtra Oxygen Consumption Assay

## Authors

Ryan McGarrigle,  
Melanie Schwalfenberg,  
Ned Jastromb, and  
James Hynes  
Agilent Technologies, Inc.

## Abstract

Mitochondria are functional regulators of life and death, and play a central role in the etiology of many diseases. Impaired mitochondrial function has been identified as a significant contributor to drug-induced toxicity and is of particular concern within the pharmaceutical industry. Oxygen consumption measurements have been the preferred method of assessing mitochondrial function. However, the measurement complexity and low-throughput nature of traditional polarographic Clark-type electrode approaches have limited the applicability of these informative measurements, particularly in compound screening. This Technical Overview describes protocols for the isolation of rat liver mitochondria and their analysis with the Agilent MitoXpress Xtra oxygen consumption assay. Protocols for setting up the assay, preparing reagents, and optimizing mitochondrial protein concentrations are also included.

## Introduction

Mitochondrial dysfunction is implicated in the etiology of multiple disease states, and has been identified as a major mechanism of drug-induced toxicity.<sup>1-6</sup> Oxygen consumption analysis has historically been the preferred means of assessing mitochondrial function because it is a direct measure of electron transport chain (ETC) activity and, in coupled mitochondria, provides specific information on oxidative phosphorylation (OXPHOS). Traditionally, oxygen consumption of isolated mitochondria has been performed using low-throughput polarographic methods. However, this approach does not provide the throughput or convenience required to assess multiple experimental conditions in parallel, whether screening compound libraries, assessing dose-response relationships, or interrogating mechanism of action.

The MitoXpress Xtra oxygen consumption assay combines information on how rapidly mitochondria consume oxygen with the convenience and throughput of fluorescence-based plate reader assays. The throughput provided, and the fact that measurement volumes are lower than conventional polarographic analysis, means that significantly larger datasets can be generated from a single mitochondrial preparation.

Many oxidizable substrates are unable to cross the plasma membrane freely, preventing investigators from controlling which substrates can enter the mitochondria. Isolating mitochondria from tissues facilitates full access of substrates to the complexes of the ETC. Consequently, direct interrogation of complexes is possible, as substrates and inhibitors can carefully be controlled. This method provides insights into the mechanisms underlying metabolic changes.

These powerful measurements are enabled by the MitoXpress Xtra probe. Fluorescence emission from the probe is quenched by molecular oxygen; therefore, reductions in dissolved oxygen concentration caused by ETC activity are observed as an increase in MitoXpress Xtra signal. To limit back-diffusion of ambient oxygen, a sealing layer of mineral oil is applied to each test well, and the plate is measured kinetically on a fluorescence plate reader to deliver a convenient high-throughput assessment of ETC activity. Decreased rates of signal change relative to an untreated control suggest inhibition of ETC activity, while increases in signal suggest increased respiration, due to either increased ETC activity or the uncoupling of ETC from OXPHOS. As with traditional polarographic approaches, a level of mechanistic insight can be provided using ETC complex-specific substrates. However, the microplate-based approach used allows all relevant conditions to be measured in parallel so that assay setups is simplified, and data interpretation is not confounded by the gradual deterioration of the mitochondrial preparation.

This technical overview describes the following protocols:

1. Preparation of reagents for use with the MitoXpress Xtra oxygen consumption assay
2. Isolation of rat liver mitochondria
3. Mitochondrial protein optimization for oxygen consumption assay
4. Screening for mitochondrial inhibitors and uncouplers
5. Screening for inhibitors of fatty acid oxidation

## Materials

### Reagents

- MitoXpress Xtra Oxygen Consumption Assay (part number MX-200-4)
- MitoXpress Mineral Oil (part number MO-200L-1)
- Male Sprague-Dawley rats (150 to 180 g; for example, Charles River)
- Sucrose (Sigma, cat. no. S7903)
- Potassium phosphate dibasic (Sigma, cat. no. P3786)
- Potassium chloride (Sigma, cat. no. P9333)
- Potassium hydroxide (Sigma, cat. no. 484016)
- Magnesium chloride hexahydrate (Sigma, cat. no. M2670)
- Ethylene-bis(oxyethylene nitrilo) tetraacetic acid (EGTA; Sigma, cat. no. E3889)
- D-Mannitol (Sigma, cat. no. M9546)
- HEPES (Sigma, cat. no. H4034)
- 4-Morpholinepropanesulfonic acid (MOPS; Sigma, cat. no. M5162)
- Bovine serum albumin-fatty acid-free (BSA; Sigma, cat. no. A7030)
- Adenosine 5'-diphosphate (ADP, Sigma, cat. no. A5285)
- Glutamate (L-glutamic acid monosodium salt hydrate; Sigma, cat. no. G1626)
- Malate (L-(-)-malic acid; Sigma, cat. no. M6413)
- Succinate (sodium succinate dibasic hexahydrate; Sigma, cat. no. S2378)
- Palmitoyl co-enzyme A lithium salt (Sigma, cat. no. P9716)
- L-Carnitine hydrochloride (Sigma, cat. no. C0283)
- BCA Protein Assay (Thermo Scientific, cat. no. 23225)

## Equipment

- Motor-driven (optional) or manual glass tissue homogenizer with Teflon pestle (55 mL; Thomas Scientific, cat. no. 3431D94)
- Glass stirring rods
- Carbon dioxide
- Sterile dissection tools
- Refrigerated centrifuge
- 96/364-well microplate, black with clear bottom
- Multichannel pipette
- Plate heater block (VWR cat. no. 12621-108)
- Water bath
- Fluorescence plate reader with temperature control (see Plate reader setup)
- Kinetic Analysis Software
- (Optional) Integra VIAFLO 384 (6031)

## Reagent preparation

- **1.0 M sucrose:** Dissolve 34.23 g of sucrose in 100 mL of distilled water; aliquot and store at  $-20\text{ }^{\circ}\text{C}$ .
- **0.5 M  $\text{MgCl}_2$ :** Dissolve 0.51 g of  $\text{MgCl}_2$  in 5 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .
- **0.1 M KCl:** Dissolve 0.75 g of KCl in 100 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .
- **0.1 M EGTA:** Dissolve 0.38 g of EGTA in 10 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .
- **0.1 M  $\text{K}_2\text{HPO}_4$ :** Dissolve 0.87 g of  $\text{K}_2\text{HPO}_4$  in 50 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .
- **1.0 M mannitol:** Dissolve 18.22 g of mannitol in 100 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .
- **0.1 M MOPS:** Dissolve 2.1 g of MOPS in 100 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .
- **0.5 M HEPES:** Dissolve 1.19 g of HEPES in 10 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$ .

- **10% BSA:** Dissolve 10 g of BSA in 100 mL of distilled water, and store at  $4\text{ }^{\circ}\text{C}$  for up to a week.
- **0.5 M glutamate/0.5 M malate:** Dissolve 1.69 g of glutamic acid and 1.34 g of malic acid in 10 mL of distilled water. Adjust pH to 7.4 with KOH, add water to bring the volume to 20 mL, aliquot, and store at  $-20\text{ }^{\circ}\text{C}$  for up to six months.
- **1.0 M succinate:** Dissolve 4.5 g of succinate in 10 mL of distilled water. Adjust to pH 7.4 with KOH, add water to make up the volume to 20 mL, aliquot, and store at  $-20\text{ }^{\circ}\text{C}$  for up to 6 months.
- **100 mM ADP:** Prepare 100 mM adenosine 5'-diphosphate in respiration buffer. Aliquot and store at  $-20\text{ }^{\circ}\text{C}$ . Keep on ice during preparation.

## Additional reagents for FAO assay:

- **Palmitoyl-CoA/carnitine/malate solution:** Prepare 1.6 mM palmitoyl CoA (16.48 mg), 80 mM carnitine (158.13 mg), and 200 mM malic acid (268.18 mg) in 10 mL of respiration buffer.

## Plate reader setup

- The user guide for MitoXpress Xtra oxygen consumption assay can be found on [agilent.com](https://www.agilent.com)<sup>7</sup>
- This application is compatible with a range of alternative fluorescence plate reader models and detection modes: 1. Dual-read time-resolved fluorescence (TRF) lifetime, 2. Single TRF intensity, or 3. Fluorescence intensity (FI) modes.
- For optimal performance, we recommend using dual-read TRF followed by single read TRF measurements as an alternative where available. Plate reader specific settings can be downloaded at <https://www.agilent.com/en/products/cell-analysis/plate-reader-compatible-assays>.

- Fluorescent plate readers are required to perform excitations at 380 nm and emissions at 650 nm using monochromator or filter-based optics.
- The temperature should be maintained at  $30\text{ }^{\circ}\text{C}$  (or  $37\text{ }^{\circ}\text{C}$ )
- The plate should be run in kinetic mode with read intervals below 2.5 minutes.
- For more information on plate reader models, detection modes, instrument settings, predefined instrument protocols, and data analysis templates (selected plate reader models), see [Pick Your Assay and Find Your Plate Reader](#).<sup>8</sup>
- We recommend that first-time users carry out signal optimization before starting the experiments. Briefly:
  1. Prepare eight replicate wells of a 96-well plate by adding 90  $\mu\text{L}$  prewarmed culture medium to each well (A1 to A4, B1 to B4).
  2. For signal control wells (S), add 10  $\mu\text{L}$  reconstituted MitoXpress Xtra reagent to four of the replicate wells (A1 to A4).
  3. For blank control wells (B), add 10  $\mu\text{L}$  water, PBS, or media to the remaining replicates wells (B1 to B4).
  4. Promptly add two drops (or 100  $\mu\text{L}$ ) of prewarmed mineral oil to all eight replicate wells, taking care to avoid air bubbles.
  5. Read the plate immediately in a fluorescence plate reader over 30 minutes (read every two to three minutes).
  6. Examine the signal control well (A1 to A4) and blank control well (B1 to B4) readings, and calculate the S:B ratio using the last reading at 30 minutes.

For fluorescence plate readers, set up accordingly, MitoXpress Xtra should return a S:B  $\geq 3$ . Higher readings are expected with TR-F and dual read TR-F measurement. If S:B is  $< 3$ , the following options may be helpful to improve the S:B ratio:

- Increase the gain (PMT) setting or flash energy.
- Adjust TR-F focal height.
- Increase the length of integration time (the same for both delay windows).
- Repeat as top or bottom read, respectively.
- Increase the concentration of MitoXpress-Xtra (1.5 $\times$ ).
- Contact the instrument supplier for further options.

Use the stock solutions to make up buffers according to Table 1. Make buffers up to 100 mL with distilled water, and adjust the pH to 7.4. Alternatively, buffers can be made by combining reagents, but ensure that the final concentration is the same. Larger volumes (>500 mL) of isolation buffer I may be required, depending on how much tissue is being homogenized.

## Isolation of rat liver mitochondria

This protocol is based on the method of Lapidus and Sokolove.<sup>9</sup> Protocols for isolation of mitochondria from rat cardiac and skeletal muscle are available.<sup>6,10</sup>

For best results, rats should be young and weigh 150 to 180 g. They should be housed in pairs in a controlled environment with constant temperature (21  $\pm$  2 °C), and a 12 hour light/dark cycle with food and water provided *ad libitum*. Additional information can be found in the Guide for Care and Use of Laboratory Animals.<sup>11</sup>

**Table 1.** Respiration buffer recipe.

| Buffer              | Reagent                         | Stock Concentration (M) | Final Concentration (mM) | Volume for 100 mL (mL) |
|---------------------|---------------------------------|-------------------------|--------------------------|------------------------|
| Respiration Buffer  | Sucrose                         | 1.0                     | 250                      | 25                     |
|                     | MgCl <sub>2</sub>               | 0.5                     | 5.0                      | 1.0                    |
|                     | KCl                             | 0.1                     | 15                       | 15                     |
|                     | EGTA                            | 0.1                     | 1.0                      | 1.0                    |
|                     | K <sub>2</sub> HPO <sub>4</sub> | 0.1                     | 30                       | 30                     |
| Isolation Buffer I  | Sucrose                         | 1.0                     | 70                       | 7.0                    |
|                     | EGTA                            | 0.1                     | 1.0                      | 1.0                    |
|                     | Mannitol                        | 1.0                     | 210                      | 21                     |
|                     | HEPES                           | 0.5                     | 5.0                      | 1.0                    |
|                     | BSA                             | 10%                     | 0.5%                     | 5.0                    |
| Isolation Buffer II | Sucrose                         | 1.0                     | 70                       | 7.0                    |
|                     | MgCl <sub>2</sub>               | 0.5                     | 10                       | 2.0                    |
|                     | EGTA                            | 0.1                     | 1.0                      | 1.0                    |
|                     | K <sub>2</sub> HPO <sub>4</sub> | 0.1                     | 5.0                      | 5.0                    |
|                     | Mannitol                        | 1.0                     | 210                      | 21                     |
|                     | MOPS                            | 0.1                     | 10                       | 10.0                   |
|                     | HEPES                           | 0.5                     | 5.0                      | 1.0                    |

### Homogenize the liver

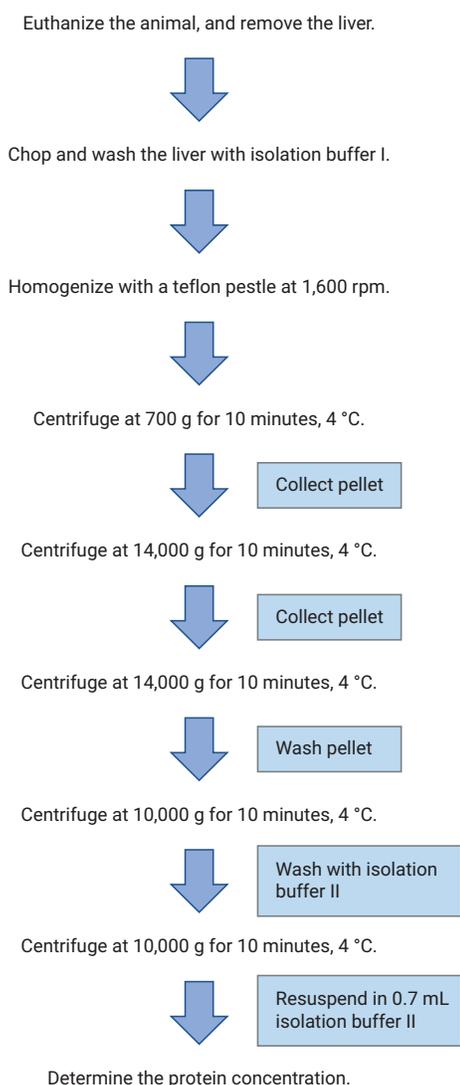
1. Euthanize animals with an overdose of carbon dioxide. Anesthetics should be avoided, as they can have adverse effects on mitochondrial quality.
2. Remove liver and place into ice-cold isolation buffer I (10 mL/g liver).
3. Chop 5 to 7 g of liver very finely with scissors. Wash the tissue several times with 20 mL of isolation buffer I until it is free of blood.
4. Homogenize the tissue in five volumes of isolation buffer I using a smooth glass grinder with a Teflon pestle driven by a power drill on low speed (six to eight passes), alternatively by hand.
5. Add eight volumes of isolation buffer I to homogenate.

### Isolate the mitochondria

1. Centrifuge the homogenate for 10 minutes at 700  $\times$  g, 4 °C, to remove debris.
2. Filter through two layers of cheesecloth, the fatty layer can be removed by aspiration.
3. Centrifuge the filtrate for 10 minutes at 14,000  $\times$  g, 4 °C, to pellet the mitochondrial fraction. Discard the supernatant.
4. Wash the mitochondrial pellet by resuspending in 20 mL of isolation buffer I using a glass stirring rod, and centrifuge for 10 minutes at 10,000  $\times$  g, 4 °C. Discard the supernatant.
5. Repeat wash step 4 using ice-cold isolation buffer II.
6. Resuspend the mitochondria in 0.7 mL of isolation buffer II, and determine the protein concentration using a protein assay (BCA).

7. Adjust to >30 mg protein/mL with isolation buffer II. Keep the mitochondria on ice, and use within four to six hours.

**Note:** Mitochondria should be freshly prepared, and should not be left on ice for longer than recommended in the literature<sup>12</sup> (typically <four to six hours for rat liver mitochondria). Freeze-thawing will disrupt mitochondrial membranes.



**Figure 1.** Procedure flowchart.

## Optimization of mitochondrial protein concentration and determination of coupling efficiency

This experiment determines the optimal concentration of isolated mitochondria for use in screening experiments and examines the coupling of the ETC to OXPHOS. The concentration of mitochondria is directly proportional to the rate of oxygen consumption, and is impacted by the respiratory substrate. To ensure robust analysis, compounds must be screened at protein concentrations that have been optimized such that strong signal changes are observed for untreated samples, so that increased and decreased activities are reliably detectable. Analysis of coupling between the ETC and OXPHOS is often useful to ensure that mitochondria are still functioning efficiently.

### Protocol

1. Equilibrate the plate reader, plate heater block, and solutions to 30 °C.
2. Prepare substrate solutions from premade stocks (Table 2).
3. Prepare a six-point dilution series of isolated mitochondria in assay buffer, total volume of 1.5 mL for each concentration.

The recommended concentrations are 1.0, 0.5, 0.25, 0.125, 0.063, and 0.03 mg/mL mitochondrial protein.

4. Resuspend MitoXpress Xtra in 1 mL of respiration buffer, then make up to 10 mL in respiration buffer.
5. Place a microplate on a plate heater block, equilibrated to 30 °C.
6. Using an automatic or multichannel pipette, add the following solutions into the pre-equilibrated 96-well plate (see Figure 1 for the recommended layout):

| 96     | 384     |                        |
|--------|---------|------------------------|
| 100 µL | 25 µL   | 1× MitoXpress Xtra     |
| 50 µL  | 12.5 µL | Optimized mitochondria |
| 50 µL  | 12.5 µL | Substrate solution     |

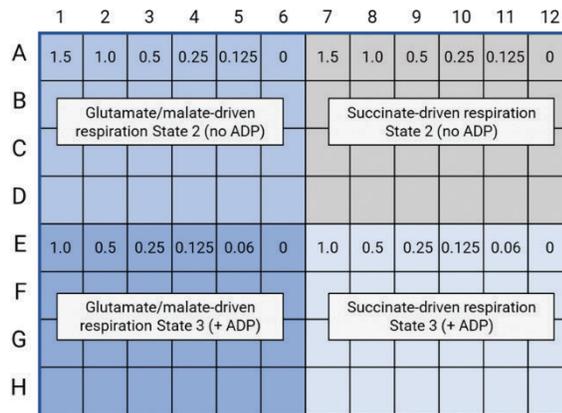
**Table 2.** Substrate preparation guide for single dose screening.

| Assay                         | Substrate Entry | Volumes  | Final Concentration     |
|-------------------------------|-----------------|--|-------------------------|
| Uncoupler Screening (State 2) | Complex I       | 150 µL Glutamate/malate + 1.35 mL Respiration buffer                   | 12.5/12.5 mM            |
|                               | Complex II      | 150 µL Succinate + 1.35 mL Respiration buffer                          | 25 mM                   |
| Inhibitor Screening (State 3) | Complex I       | 150 µL Glutamate/malate + 100 µL ADP Stock+ 1.25 mL Respiration buffer | 12.5/12.5 mM<br>1.65 mM |
|                               | Complex II      | 150 µL Succinate + 100 µL ADP Stock+ 1.25 mL Respiration buffer        | 25 mM<br>1.65 mM        |

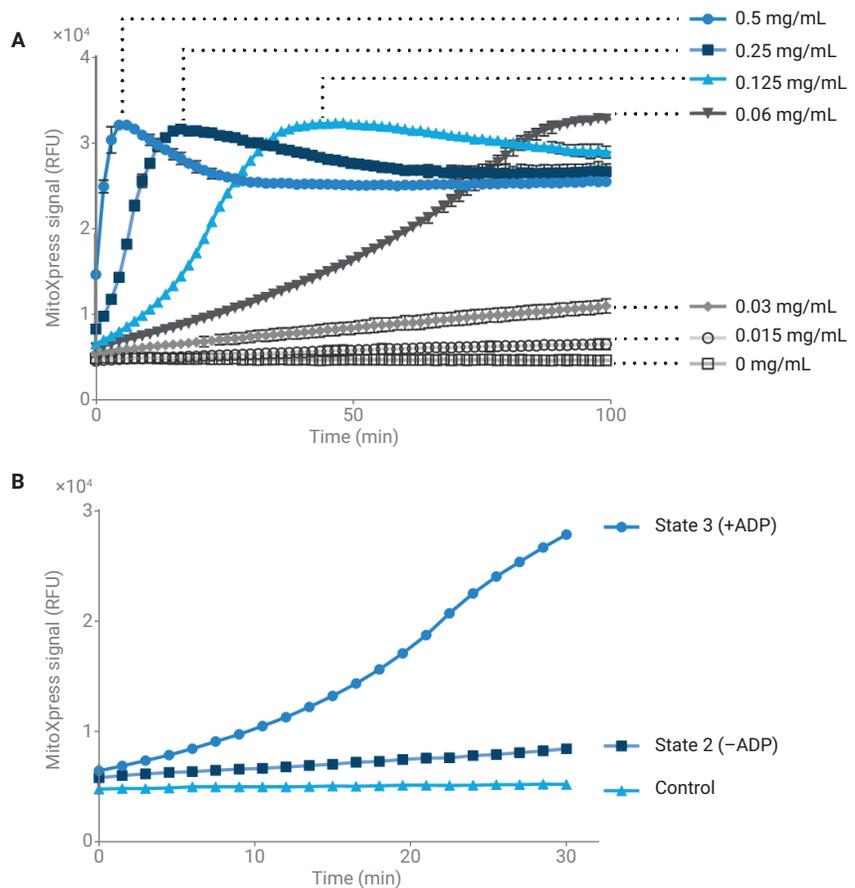
- Seal the plate using a syringe dispenser, quickly add 100  $\mu\text{L}$  (or 50  $\mu\text{L}$  for 384-well) prewarmed mineral oil to each well, taking care to avoid air bubbles (optional: liquid handling step in the Appendix).
- Insert the microplate into the prewarmed plate reader, and commence reading.
- Analyze kinetic data by taking the slopes of the linear portions of signal curves. Normalize to the untreated control (See Data analysis).
- Compare samples with ADP (State 3) and without ADP (State 2) for each individual substrate to obtain the State 3/State 2 ratio. See Figure 2 for raw sample data.

The ratio of State 3/State 2, the respiratory control ratio (RCR), is a measure of coupling, and is an important indicator of the quality of the mitochondrial preparation.

Screens are typically performed at 0.5 mg/mL succinate or 1 mg/mL glutamate/malate for basal respiration, or at 0.25 mg/mL succinate or 0.25 mg/mL glutamate/malate for ADP-driven respiration.



**Figure 1.** Plate layout for mitochondrial optimization and RCR determination. Each group on the 96-well example contains protein titration for each of the two substrates with and without ADP.



**Figure 2.** (A) Example of signal curves from protein titration for ADP-driven respiration with succinate (from 0 to 0.5 mg/mL). (B) Impact of ADP addition on mitochondrial oxygen consumption.

## Compound screening

This protocol outlines the procedure for testing the effect of compounds with potential mitochondrial liabilities on isolated mitochondria from rat liver. Mitochondria from other tissues can also be used with this protocol, however optimization steps should be repeated. This Technical Overview describes how compounds can be screened initially for a yes/no effect at a single concentration, or for interrogation by dose-response.

1. Aliquot stock solutions of test compounds in a 96-well plate according to the plate maps in Figure 3. Dilute compounds with measurement buffer I to produce known concentrations as follows:

For initial screening (yes/no effect): Assay at a single concentration (for example, 100 nmol/mg) in duplicate (Figure 3A).

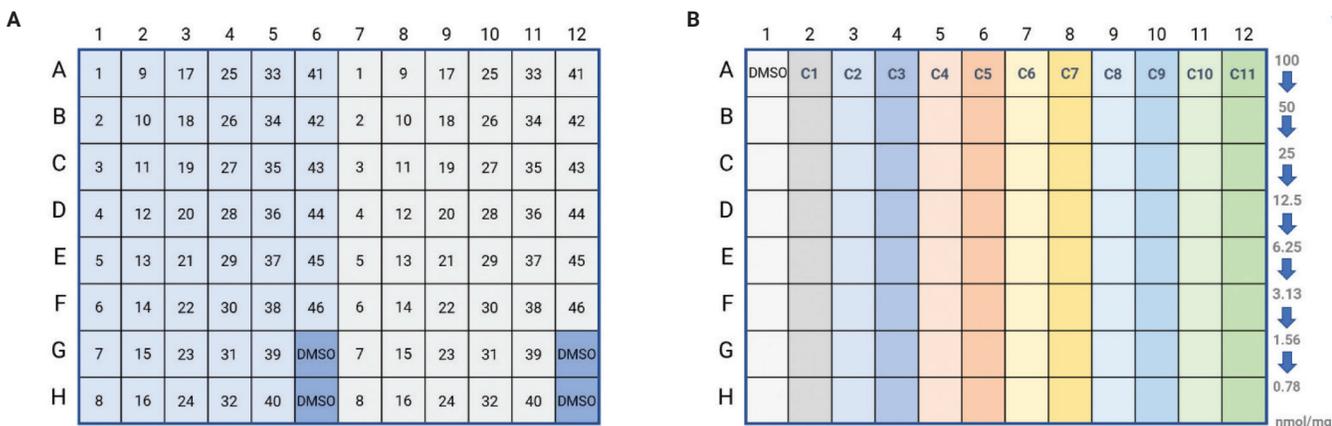
For analysis of hits and generation of  $IC_{50}$  values: Prepare serial 1:2 dilutions for each compound at one data point per concentration (Figure 3B). As drug concentrations are usually expressed in nmol/mg mitochondrial protein, different dilutions need to be prepared depending on the chosen mitochondrial test concentration. The final DMSO content in the assay wells should not exceed 0.5% (v/v).

2. Prepare substrate solutions from prewarmed stocks according to Table 3.
3. Prepare 6 mL of mitochondria solution at the optimal working dilution (previously determined using the optimization protocol).
4. Place a microplate on a plate block heater equilibrated to 30 °C and, using an automatic or multichannel pipette, dispense the following solutions into the pre-equilibrated 96-well plate (see Figure 3 for recommended layout):
5. Using a syringe dispenser, quickly add 100  $\mu$ L (or 50  $\mu$ L for 384-well) prewarmed mineral oil to each well, taking care to avoid air bubbles. (optional: liquid handling step in the Appendix)
6. Insert the microplate into the prewarmed plate reader, and commence reading.
7. When the measurement cycle is complete, remove the plate from the instrument, and save the data to file.
8. Analyze kinetic data by taking the slopes of the linear portions of signal curves. Normalize to the untreated control (See Data analysis).

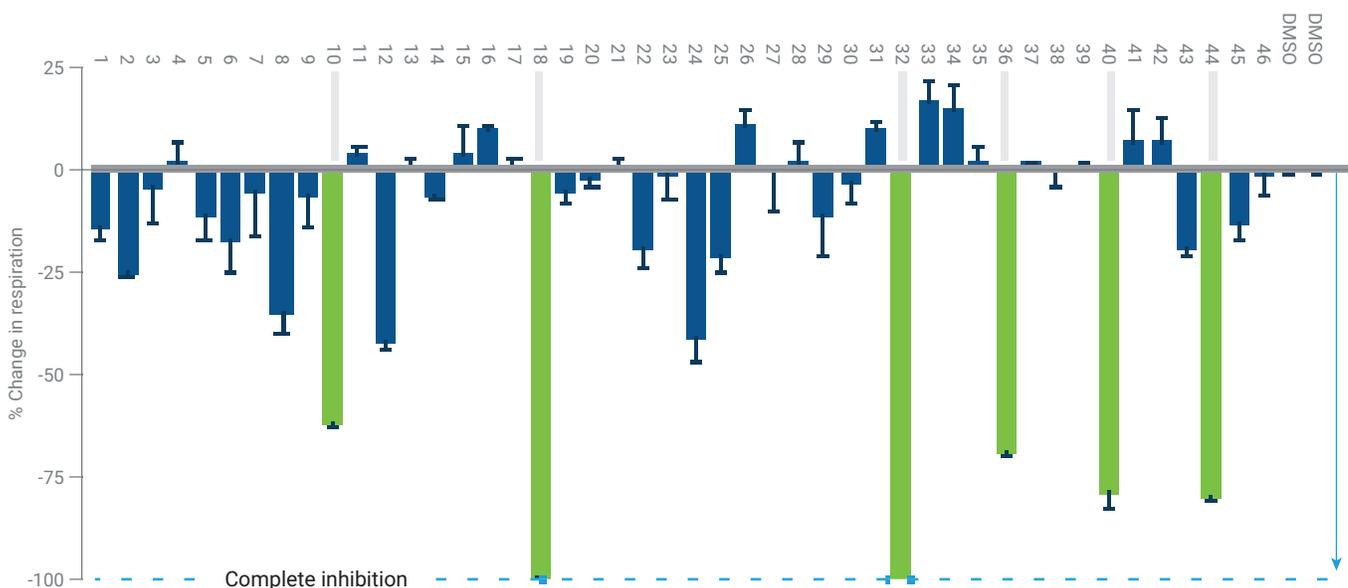
| 96          | 384          |   |
|-------------|--------------|---|
| 100 $\mu$ L | 25 $\mu$ L   | 1 $\times$ MitoXpress Xtra                    |
| 50 $\mu$ L  | 12.5 $\mu$ L | Optimized mitochondria                        |
| 50 $\mu$ L  | 12.5 $\mu$ L | Substrate solution                            |
| 2 $\mu$ L   | 1 $\mu$ L    | Test compound (for 384-well use 50 $\times$ ) |

**Table 3.** Substrate preparation guide for dose-response.

| Assay                         | Substrate Entry | Volumes   | Final Concentration  |
|-------------------------------|-----------------|---|----------------------|
| Uncoupler Screening (State 2) | Complex I       | 600 $\mu$ L Glutamate/malate + 5.4 mL Respiration buffer                        | 12.5/12.5 mM         |
|                               | Complex II      | 600 $\mu$ L Succinate + 5.4 mL Respiration buffer                               | 25 mM                |
| Inhibitor Screening (State 3) | Complex I       | 600 $\mu$ L Glutamate/malate + 400 $\mu$ L ADP Stock+ 5.0 mL Respiration buffer | 12.5/12.5 mM 1.65 mM |
|                               | Complex II      | 600 $\mu$ L Succinate + 400 $\mu$ L ADP Stock+ 5.0 mL Respiration buffer        | 25 mM 1.65 mM        |

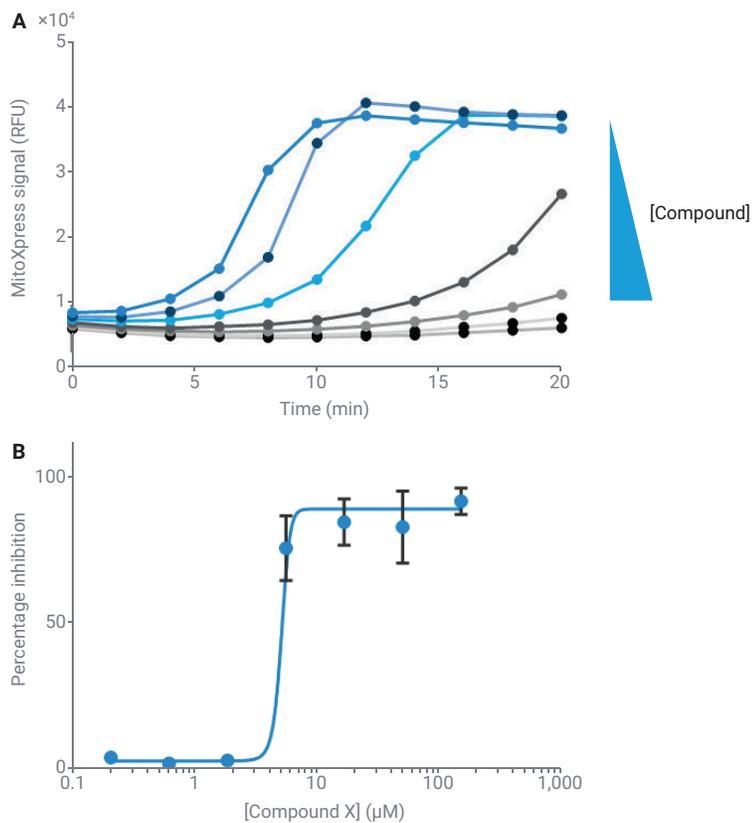


**Figure 3.** Plate layout for yes/no screening (A) and for dose-response analysis (B).



**Figure 4.** Forty-six compounds can be screened in duplicate on one plate using the plate layout presented in Figure 2A. Two vehicle controls (DMSO) are included on the plate. Data are presented as the change in percentage inhibition (B). Compounds showing significant inhibition are flagged and the data transposed for dose-response analysis (highlighted in green).

9. To generate a dose-response curve, plot the generated data against the corresponding compound concentration. For inhibitors, apply sigmoidal fits, or plot it to determine  $IC_{50}$  values (for inhibitors) or  $UC_{50}$  values (for uncouplers). See Figure 5B for an example.
10. Group compounds based on their effect on mitochondria, and rank their order based on  $UC_{50}$  or  $IC_{50}$  values.



**Figure 5.** (A) Representative signal curves from a dose response of a compound of interest (B) Representative  $IC_{50}$  curve from a mitochondrial inhibitor based on measured slopes. Data were normalized to untreated control rates.

## Appendix

### Fatty acid oxidation screening

This protocol outlines how the screening assay can be adapted to test fatty acid oxidation in isolated rat liver mitochondria. Potential fatty acid oxidation inhibitors can be screened using palmitoyl-CoA as a substrate. Oxygen consumption of isolated mitochondria in State 3 (+ADP) using palmitoyl-CoA/carnitine/malate, as respiratory substrates is expected to be reduced in the presence of inhibitors of fatty acid oxidation or ETC inhibitors.

Under these conditions, further delineation can be achieved by adding glutamate/malate. Glutamate can restore oxygen consumption by bypassing the fatty acid oxidation pathway and feeding electrons into complex I, through the generation NADH following the conversion of glutamate to alpha-ketoglutarate. If oxygen consumption is restored in the presence of glutamate/malate, this suggests specific inhibition of fatty acid oxidation; whereas inhibition under both conditions is indicative of a specific or multifactorial inhibition of the ETC.

1. Thaw substrate stock solutions (palmitoyl-CoA/carnitine/malate and glutamate/malate) on ice. Equilibrate Respiration Buffer and oil to 30 °C
2. Reconstitute MitoXpress Xtra in 1 mL of Respiration Buffer, and mix to ensure resuspension. Then, dilute to 11 mL with Respiration Buffer, and warm to 30 °C.
3. Add 100 µL Respiration Buffer/MitoXpress to each well.
4. Add 2 µL of 100× compounds dissolved in DMSO to well test well and 1 µL DMSO to control wells.

For initial screening, test each compound at a single concentration (typically, 100 nmol/mg). See Figure 3A for plate map.

For subsequent mechanistic elucidations and dose-response analysis, test each compound at multiple concentrations. See Figure 3B for plate map.

5. To test both (a) palmitoyl-CoA/carnitine/malate plus ADP and (b) palmitoyl-CoA/carnitine/malate plus ADP + glutamate/malate, add:
  - For palmitoyl-CoA/carnitine/malate plus ADP as substrates
    - i. 600 µL of palmitoyl-CoA/carnitine/malate substrate stock
    - ii. 400 µL ADP
    - iii. Optimized mitochondria (typically, ~0.5 to 1 mg/mL\*)
    - iv. Make up to 11 mL with Respiration Buffer
  - For palmitoyl-CoA/carnitine/malate + glutamate/malate plus ADP
    - i. 600 µL of palmitoyl-CoA/carnitine/malate substrate stock
    - ii. 600 µL glutamate/malate
    - iii. 400 µL ADP
    - iv. Optimized mitochondria (typically, ~0.25 to 0.5 mg/mL\*)
    - v. Make up to 11 mL with Respiration Buffer
6. Using a multichannel pipette, dispense 100 µL in each well as quickly as possible.
  - \* The protein concentration is highly dependent on the quality of the mitochondrial preparation, and therefore will need to be optimized by the user specifically for these substrates.
7. Dispense 100 µL of prewarmed mineral oil into each well.
8. Insert the 96-well plate into the fluorescence plate reader, and start the measurement.

### Data analysis

Basic analysis is the most common approach taken. Use the instrument's data analysis software to plot time profiles for each well. If such software is unavailable, export data using the settings found in the Agilent Data Visualization Tool user guide.<sup>13</sup> Select the linear portion of the signal profile (avoiding any initial lag or subsequent plateau), and apply linear regression to determine the slope for each well. Tabulate the slope values for each test sample, calculating the appropriate average and standard deviation values across replicate wells.

Percentage activity can be calculated by normalizing to untreated controls:

- Percentage activity = (Rate of treated sample/Rate of untreated control) × 100
- IC<sub>50</sub>/UC<sub>50</sub> curves plot percentage activity versus compound concentration (X-axis is shown as a Log 10 scale)

Where available, advanced analysis is performed using radiometric (Dual Read) TR-F data. Oxygen conversion is possible on some instrument analysis packages. If your instrument is not supported by the Data Visualization Tool, the lifetime can be calculated manually using blank-corrected intensity values as follows:

Lifetime (µs)[τ] = (D<sub>2</sub> - D<sub>1</sub>)/ln(R<sub>1</sub>/R<sub>2</sub>), where R<sub>1</sub> and R<sub>2</sub> represent read one and read two.<sup>7</sup> This provides lifetime values with units of µs. These lifetime profiles can be converted to an oxygen scale using a default calibration function:

$$O_2\% = 522.8 \times (-\tau/9.064)$$

Data linearization can also be performed.<sup>14</sup>

## 384-well oil dispensing

It is possible to adapt this protocol for use with liquid handling and automation systems. This study describes a method for rapid oil dispensing using a VIAFLO 384 system, kindly developed and provided by Min Tseng and Tomo Kiyota, Investigative Toxicology, Department of Safety Assessment, Genentech, Inc. This step reduces individual pipetting of oil on to a 384-well plate to a single dispensing step.

1. Load the mineral oil into an Integra 300 mL Automation Friendly Reservoir (6307), (a leftover oil can be stored here with the lid on). Replenish the oil before the level gets too low.
2. Attach 5 to 125  $\mu$ L of XYZ GRIPTIPS (6465) to the VIAFLO 384 pipetting head.
3. On the VIAFLO control panel, select the **Pipet** program.
4. Enter the following settings:
  - Volume = 25  $\mu$ L
  - Aspirating Speed = 2
  - Dispensing Speed = 1 (the slowest)
  - No predispensing or no postdispensing
5. Place the plate on a prechilled plate holders/blocks on the VIAFLO system.
6. When ready to load oil, manually move the control arm in the Z direction (up-down), and submerge part of the tip in the oil reservoir, press **Aspirate**, and hold the arm for 10 to 15 seconds while still submerged in the oil to allow the oil level to rise inside the tip. Once the levels stabilize, gently pull the arm up, and move over to the assay plate.

7. Have the tip hanging above the center of the well (~1 mm) without touching, to prevent air bubbles, load the oil onto the plate as hanging droplets. Usually, 25  $\mu$ L of oil is enough for two full droplets.
8. After loading, check for air bubbles. If there are any air bubbles, gently remove them.
9. Load the assay plate to the plate reader.

## Troubleshooting

### Mitochondrial isolation

Low activity or poor RCR values, can be caused by using animals that are too old, contaminated glassware (leading to uncoupling or inhibition), or overly harsh digestion or homogenization conditions. Activity diminishes in less than four to six hours. This can be caused by mitochondria being stored at <30 mg/mL, not being kept on ice, or being excessively handled.

### Signals are indistinguishable from blanks

To ensure that a specific probe signal is being detected, check the instrument suitability and setup, and run proper controls with and without a probe.

### Signals are detectable, but signal changes are small

Ensure that oil has been applied. If signal changes are still too small, try to increase the concentration of the mitochondria in the well.

### Declining signal over the initial 10 minutes of measurement

This is caused by the plate heating from room temperature to the measurement temperature, and can be eliminated by preparing the plate on a plate heater and prewarming all solutions. Alternatively, one can conduct data analysis after the control signal has stabilized.

A temperature of 30 °C may be better than 37 °C, as the temperature can be more stable at 30 °C.

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