

# Screening for Drug-Induced Mitochondrial Dysfunction Using MitoXpress Xtra on Isolated Mitochondria

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## Abstract

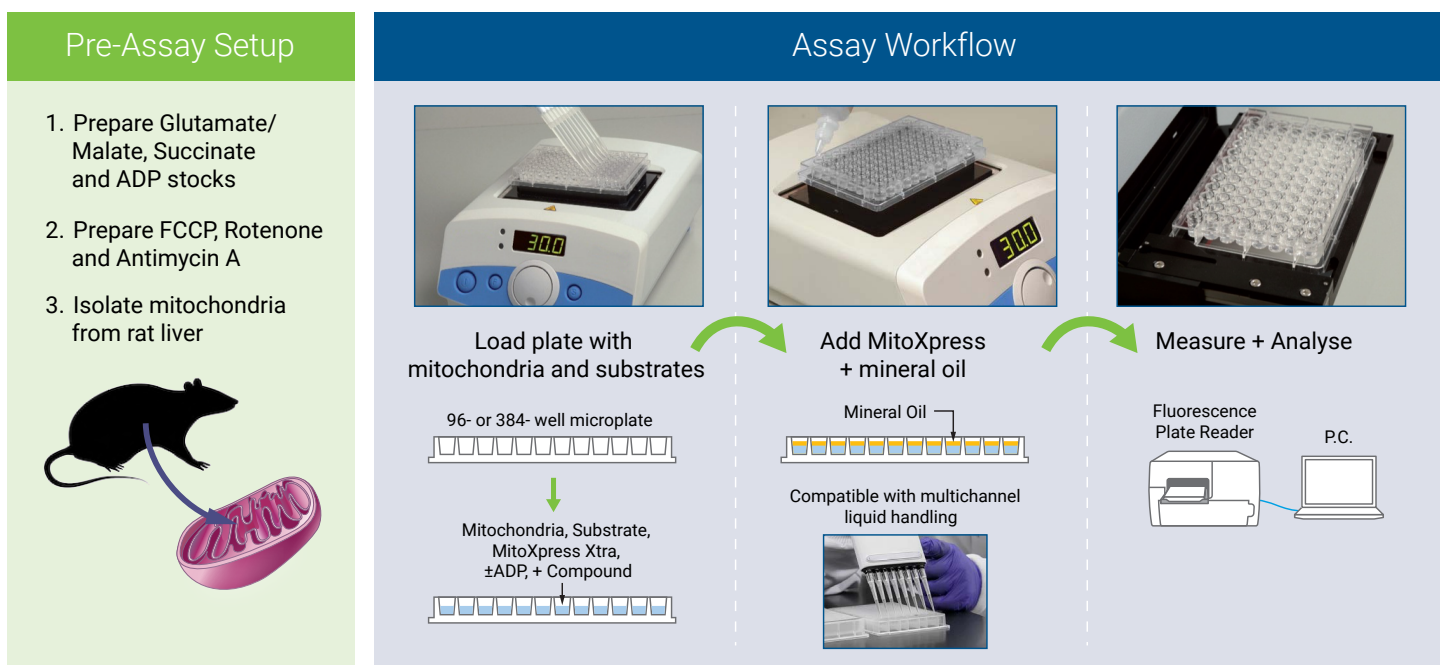
Mitochondria are functional regulators of life and death, and therefore play a central role in the etiology of many diseases. Impaired mitochondrial function has also been identified as a significant contributor to drug-induced toxicity and is therefore of particular concern within the pharmaceutical 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 the context of compound screening. Here we describe how Agilent's MitoXpress Xtra Oxygen Consumption assay can address this deficit, facilitating a fluorescence-based high-throughput interrogation of mitochondrial oxygen consumption in 96- or 384-well plate formats. Consideration is given to key optimization parameters and sample data is described, illustrating how compound libraries can be screened and dose-response analyses performed.

## Introduction

Mitochondrial dysfunction is implicated in the etiology of multiple disease states and has also been identified as a major mechanism of drug-induced toxicity [1-6]. Oxygen consumption analysis has historically been the preferred means by which mitochondrial function is assessed as 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. Here we describe how 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.

These powerful measurements are enabled by the MitoXpress Xtra sensor. Sensor emission is quenched by molecular oxygen, therefore reductions in dissolved oxygen concentration that are caused by ETC activity is observed as an increase in MitoXpress Xtra signal. A sealing layer of mineral oil is applied to each test well to limit back diffusion of ambient oxygen 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. Additionally, as with traditional polarographic approaches; a level of mechanistic insight can be provided by using ETC complex-specific substrates. However, the microplate-based approach used here allows all relevant conditions to be measured in parallel so that assay set-ups are simplified, and data interpretation is not confounded by gradual deterioration of the mitochondrial preparation.

Here we summarise how these measurements are performed, focusing particularly on key optimization parameters and on the application of such measurements to compound screening and dose-response analyses.

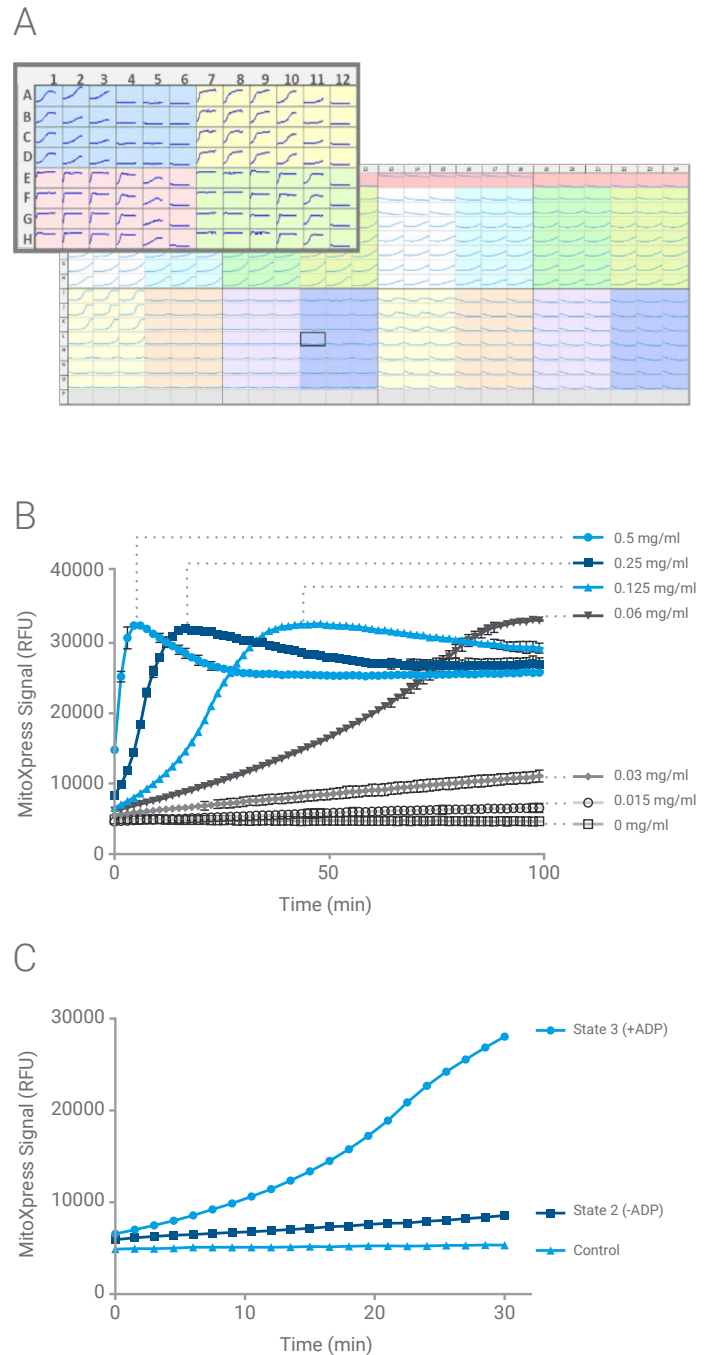


## Assay Optimization

### Optimization of Mitochondrial Protein Concentration

Oxygen consumption per mg of mitochondrial protein is tissue-specific and can also be impacted by the isolation procedure used. Oxygen consumption is also impacted by the respiratory substrates provided, and the availability of ADP. 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 both increased and decreased activities are reliably detectable. In contrast to traditional polarographic analyses; the throughput facilitated by microplate format (96 & 384) used here makes such optimization very straightforward.

Sample data is presented in Figure 1 whereby protein dilutions are assessed with relevant respiratory substrates, in the presence and absence of ADP. Increasing mitochondrial protein concentration results in more rapid oxygen depletion, detected in real-time as a more rapid increase in probe signal. As evident in Fig. 1A, the throughput enabled by the microplate approach allows multiple conditions to be assessed in a single plate.



**Figure 1.** (A) Examples of plate layout and resulting signal curves for 96- and 384-well formats. Each group on the 96-well example contains protein titration with the following substrates Glutamate/malate (Blue), Succinate (Yellow), Glutamate/malate + ADP (Pink) and Succinate + ADP (Green). (B) Example of signal curves from protein titration for ADP-driven respiration with succinate (from 0 - 0.5 mg/ml). (C) Impact of ADP addition on mitochondrial oxygen consumption.

Typical optimized protein concentrations are as follows:

- Basal Respiration glutamate/malate: 1 mg/ml
- ADP-driven respiration with glutamate/malate: 0.125 mg/ml
- Basal Respiration with succinate: 0.5 mg/ml
- ADP-driven respiration with succinate: 0.25 mg/ml

Figure 1B shows an example of signal changes from the protein optimization of ADP-driven respiration with succinate (wells E7-H12). A concentration of 0.25 mg/ml shows rapid yet measurable signal change, ideal for investigating compounds suspected of inhibiting complex II-IV of the ETC.

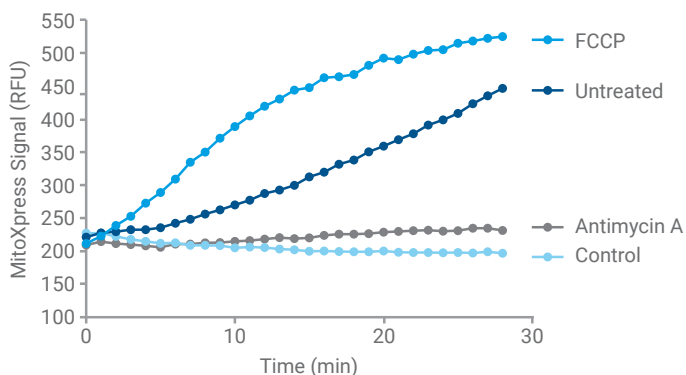
Such optimization also facilitates an assessment of mitochondrial coupling, which informs on the quality of the mitochondrial preparation. This is assessed by determining the ratio of ADP-stimulated rates (State 3) to substrate-only rates (State 2). The higher this Respiratory Control Ratio (RCR) the better the coupling of ETC activity to ATP production, contrary to low values which are indicative of damaged mitochondrial membranes. Sample data is provided in Fig. 1C illustrating the increase in activity caused by ADP addition.

### Measuring Perturbed Mitochondrial Function

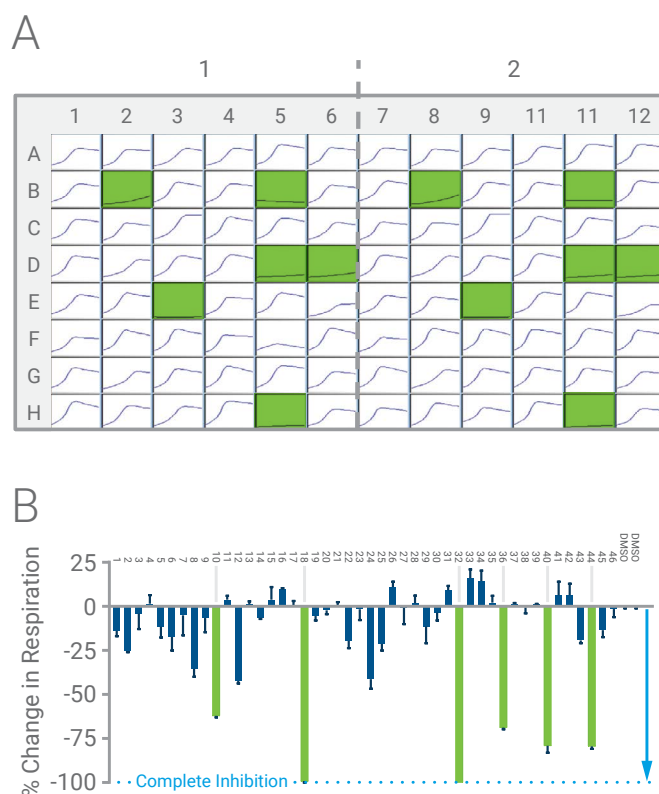
Typical data illustrating perturbed mitochondrial function mitochondria is shown in Fig. 2. Antimycin A blocks complex III of the electron transport chain, thereby inhibiting oxygen consumption, while the classical uncoupler FCCP dissipates the mitochondrial membrane potential causing an increase in oxygen consumption.

For compound screening, measurements are typically performed in State 2 (no ADP) when screening for uncouplers, and in State 3 (ADP added) when screening for inhibitors. The microplate format facilitates significant throughput with measurements possible on both 96- and 384-well formats. This is in marked contrast to traditional low-throughput polarographic approaches.

Fig. 3 illustrates how 46 compounds can be screened in duplicate on a single 96-well plate, compounds are added in parallel wells on each of half the plate. Significant decreases in respiration are easily identified (highlighted in green).



**Figure 2.** Typical data output illustrating the detection of perturbed mitochondrial function using MitoXpress Xtra.

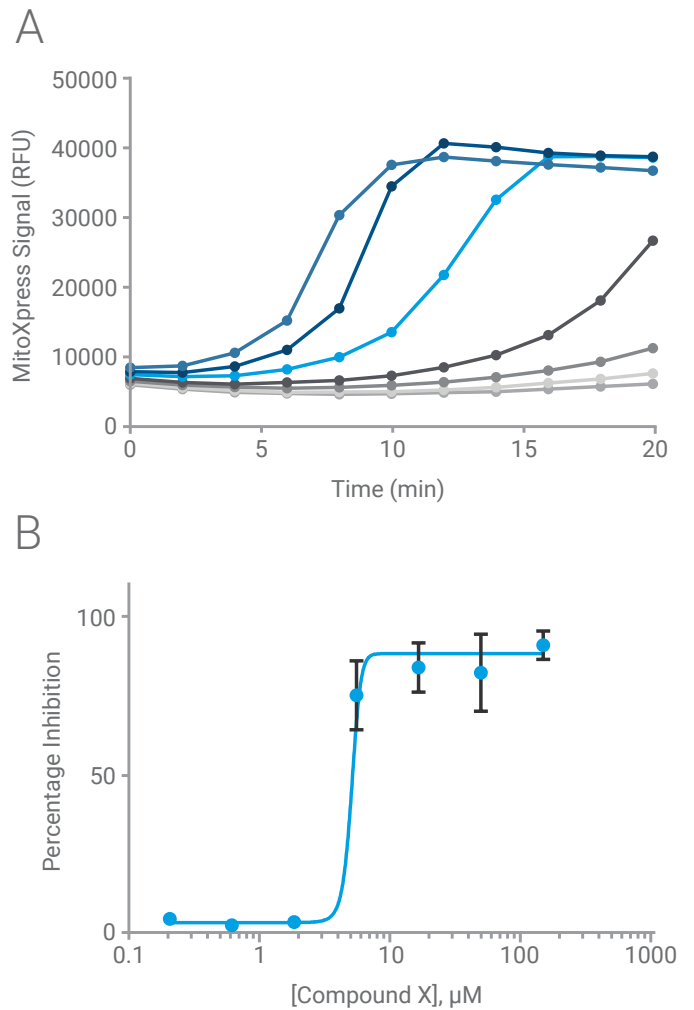


**Figure 3.** 46 Compounds can be screened in duplicate on one plate using the plate layout presented in A. Two vehicle controls (DMSO) are included on the plate. Data is presented as the change in percentage inhibition (B). Compounds showing significant inhibition are flagged and the data transposed for dose response analysis (highlighted in green).

Convenient dose-response assessments for multiple compounds can also be performed on a single plate with data easily transposed into  $IC_{50}$  /  $UC_{50}$  values, rate data from signal curves was plotted against concentrations used in the dose-response (Fig. 4B). Results can then be compared to reference literature or in-house data.

### Increasing throughput

MitoXpress Xtra based measurements of isolated mitochondria are compatible with 384-well plate formats allowing for an additional 4-fold increase in throughput. Additional throughput can be further facilitated by incorporating liquid handling approaches including single step 384-channel pipettes and basic automation systems. The assay allows for mitochondria to be used directly after isolation without plate preparation and assay times are typically ~30-45 min allowing for multiple experiments per day/preparation.



**Figure 4.** (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 was normalized to untreated control rates.

## Materials and Methods (Protocol)

### 96-well Plate Preparation

- Prepare plates on a plate heater equilibrated to 30°C.
- Reconstitute MitoXpress Xtra probe in 1 ml H<sub>2</sub>O. Dilute 1:10 in measurement buffer (250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM MgCl<sub>2</sub>, 30 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and add 100 µl to each well.
- For compound testing, add 1 µl of compound in an appropriate solvent to test wells.
- Dilute Mitochondria to the desired concentration in measurement buffer and add 50 µl to test well. Dissolve substrate (succinate or glutamate/malate) and ADP (if required) in measurement buffer and add 50 µl of this solution to test wells giving a final substrate concentration of 25 mM (succinate) or 12.5/12.5 mM (glutamate/malate) and a final ADP concentration of 1.65 mM.
- Add 100 µl of mineral oil (preheated to 30°C) to each well. This increases assay sensitivity by minimizing interference from ambient oxygen.

Note: Plate preparation time should be kept to a minimum. If using 384-well plate adjust volumes accordingly. (See Additional technical resources)

### Measurement

- Insert the prepared plate into a fluorescence plate reader pre-set to 30°C.
- Measure sensor signal at 1.5 min intervals for 10-30 min using excitation and emission wavelengths of 380 nm and 650 nm respectively as per user manual.
- Fluorescence intensity of the MitoXpress Xtra probe was measured kinetically using a fluorescence plate reader. (Settings for selected readers can be requested from [cellanalysis.support@agilent.com](mailto:cellanalysis.support@agilent.com)) or available in the Instrument Guide[11].
- Intensity signal (RFU) were then plotted versus time as described in [10], in this instance using preconfigured BMG Data Analysis Template (can be requested from [cellanalysis.support@agilent.com](mailto:cellanalysis.support@agilent.com)).

Where available, time-resolved fluorescence (TRF) should be used to maximize measurement performance. Recommended TRF measurement settings are provided in the MitoXpress Xtra user manual. Instrument protocols available from [agilent.com](http://agilent.com) (see additional resources).

Note: Mitochondria are freshly prepared as per user's protocol and should not be left on ice for longer than recommended in the literature [9] (typically <4-6 h for rat liver mitochondria). Measurement buffers should be prepared freshly on the day of measurement.

### Data Analysis

Data is typically interrogated in a semi-quantitative manner by determining the rate of MitoXpress Xtra sensor signal increase, this is used to compare treated to untreated samples and to generate dose-response relationships.

Rates can be generated by taking the slopes of the linear portions of kinetic curves. Percentage activity can be calculated by normalizing to untreated controls:

$$\text{Percentage activity} = (\text{Rate of treated sample} / \text{Rate of untreated control}) \times 100.$$

IC<sub>50</sub> / UC<sub>50</sub> curves plot Percentage activity vs Compound Concentration (x-axis is shown as a Log 10 scale).

Although not typically required, a more quantitative assessment can be performed by transposing fluorescence data into oxygen concentrations [9].

## Conclusion

The MitoXpress Xtra Oxygen Consumption Assay facilitates a convenient, sensitive, direct interrogation of mitochondrial function. As this fluorescence-based assay is compatible with 96- and 384-well plates, it delivers the throughput required for both compound screening and dose response assessments. All conditions are prepared in parallel such that no additional interventions are required during measurement thereby providing a simple, scalable assay workflow. Additionally, short measurement times (<30 min), high throughput, and low measurement volumes (<150 µl) maximize the amount of data that can be generated from a single mitochondrial preparation. Delivering these solutions coincides with increasing adoption of these reagents in both academic and drug discovery settings ultimately allowing for the screening of drugs for potential mitochondrial liabilities to aid in assay design and avoiding toxicity.

## Additional Technical Resources

- Relevant Publications: [6-9]
- MitoXpress Xtra Oxygen Consumption Assay User Manual [10]
- Protocol for mitochondrial isolation and measurement of with MitoXpress Oxygen Consumption Assay
- Instrument Set-up Guide: Data Sheets [11]
- Webinar: Challenges in Assessing Mitochondrial Toxicities and Liabilities in Drug Discovery [12]

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Printed in the USA, March 8, 2019  
5994-0784EN

