

Principle of Mitochondrial Toxicity Assessment Using Agilent Seahorse XF Solution

Authors

George W. Rogers,
Lisa Winer,
Melanie Schwalfenberg,
Natalia Romero, and
Yoonseok Kam
Agilent Technologies, Inc.

Abstract

Agilent Seahorse XF analyzers are used to study cellular metabolism in a diverse array of research areas, including assessment of drug-induced mitochondrial toxicity. Direct measurement of mitochondrial oxygen consumption can be used as a specific and sensitive indicator to assess drug-induced mitochondrial toxicity, a common issue in therapeutic development. This white paper establishes a customized XF assay solution for the detection of mitochondrial toxicity using standardized parameters. The solution includes the following key features:

- The ability to both identify and distinguish among different modes of mitochondrial toxicity
- A standardized quantitative measurement of the magnitude of the toxicity
- A metric of assay quality and performance evaluation to establish confidence in resulting data
- A rapid, straightforward assay set up for either compound screening or dose-response analysis

This white paper outlines the approach and design of the Agilent Seahorse XF Mito Tox assay solution with respect to the elements listed above, with a focus on the derivation of the mitochondrial toxicity index (MTI), a new standardized parameter enabling intuitive and quantitative assessment of the magnitude and type of mitochondrial toxicity.

Introduction

Mitochondrial toxicity is a common issue with therapeutic development, as most eukaryotic cells use mitochondria to produce the majority of adenosine triphosphate (ATP) required for metabolic function, and to regulate key cellular processes. Drugs can disrupt mitochondrial function in many ways (Figure 1) by inhibition of: electron transport chain (ETC) protein complexes, ATP synthase and other oxidative phosphorylation (OxPhos) components, enzymes of the tricarboxylic acid (TCA) cycle, various mitochondrial transporters, mitochondrial transcription and translational machinery, as well as by inhibition through the uncoupling of the ETC from ATP synthesis.¹

Much progress has been made in the past fifteen years to develop a variety of high-throughput, applicable preclinical organelle-based and *in vitro* cell models, with oxygen consumption-based methods being described as the most informative and sensitive among assays used to assess mitochondrial dysfunction and toxicity.²⁻⁴ Among the methods, direct measurement of mitochondrial oxygen consumption using Agilent Seahorse XF technology has been well documented as a specific and sensitive marker/indicator.^{1,2}

To provide an accessible and rigorous methodology for researchers who develop drugs, a customized assay kit, the Agilent Seahorse XF Mito Tox Assay Kit, was developed with dedicated workflow and analytic tools. This assay kit offers a standardized solution to identify mitochondrial toxicant compounds via direct assessment of changes in mitochondrial function. The XF Mito Tox assay design uses a modified injection strategy and automates key data calculations. In combination with the XF Pro platform, software, and consumables, this solution provides information-rich data with high sensitivity, specificity, and improved throughput using a newly defined metric, the MTI, allowing rapid and accurate interpretation of data. In addition, a metric of assay performance, Z', is used to establish and maintain high confidence in resulting data.

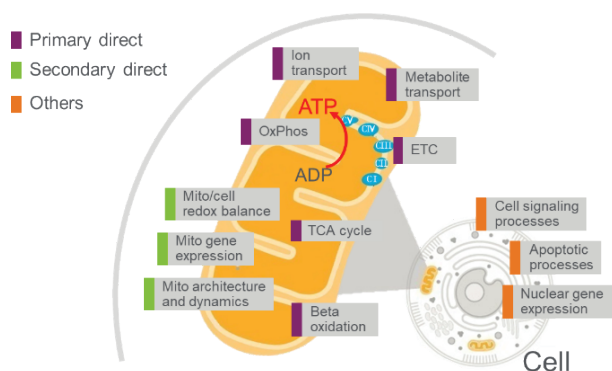


Figure 1. Key mitochondrial and cellular processes that are potential targets of drug-induced mitochondrial toxicity. Primary direct targets include metabolite and ion transport, TCA, fatty acid oxidation (FAO), ETC, and OxPhos machinery. Secondary direct targets include redox balance, mitochondrial gene expression, architecture, and dynamics. Drugs affecting other upstream processes, such as cell signaling, apoptosis, and nuclear gene expression, may also result in downstream mitochondrial toxicity. This figure is meant for illustrative purposes and is not an exhaustive list.

Approach

In the XF Mito Tox assay solution, the XF Mito Tox Assay Kit measures real-time mitochondrial function and the software features are customized for mitochondrial toxicity test to:

- Identify mitochondrial toxicity accurately and consistently with high sensitivity
- Specifically distinguish among different modes of mitochondrial toxicity
- Provide a standardized quantitative measurement of the magnitude of the toxicity
- Evaluate assay quality and establish confidence in resulting data
- Provide rapid, straightforward assay design and intuitive data interpretation for either compound-screening or dose-response assays

Assay design

XF measurement and injection strategy

The XF Mito Tox assay is a customized assay for assessing mitochondrial toxicity. This assay allows evaluation of both the type and magnitude of mitochondrial toxicity due to compound treatment. Seahorse XF technology provides standard solutions for the functional analysis of mitochondrial respiration by directly measuring OCR in real time using live cells, typically using a series of injections of reference modulators including oligomycin (OxPhos inhibitor), FCCP (uncoupler), and rotenone/antimycin A (Rot/AA, ETC inhibitors). In the XF Mito Tox assay, consecutive injections of oligomycin and FCCP are used, in combination with two control groups pretreated with vehicle and Rot/AA (Figure 2A).

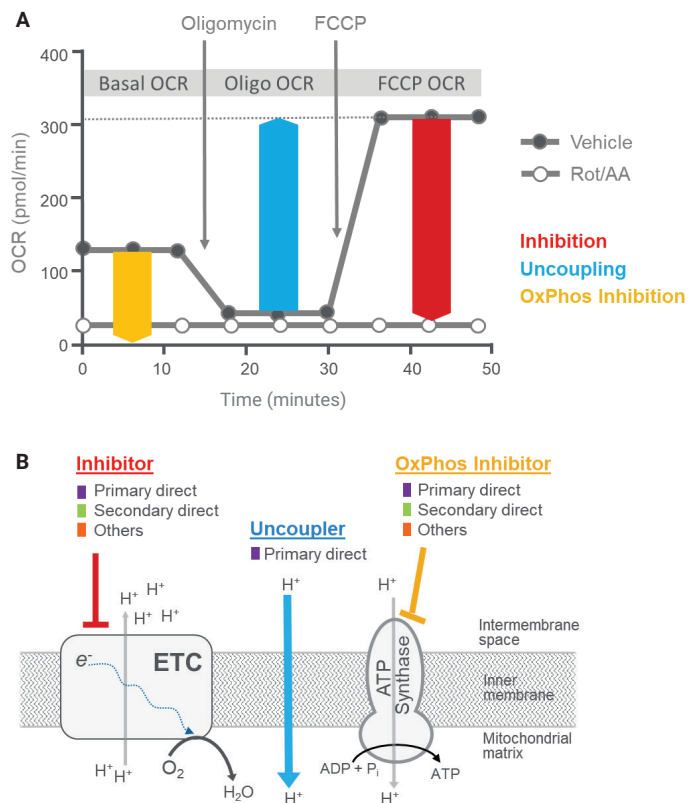


Figure 2. Modes of mitochondrial toxicity identified by the Agilent Seahorse XF Mito Tox assay. (A) Assay kinetic graph illustrating control groups (vehicle and Rot/AA) with oligomycin and FCCP injections. The effect of inhibitors, uncouplers, and OxPhos inhibitors (OPIs) are detected by measuring changes in FCCP OCR, Oligo OCR, and Basal OCR, respectively. (B) Compounds that exert effects on transport, TCA, FAO, ETC, or other upstream processes that result in decreased FCCP OCR are categorized as inhibitors. Compounds that act as protonophores that uncouple the ETC from the OxPhos machinery that result in increases in Oligo OCR are categorized as uncouplers. Compounds that specifically result in suppression of the OxPhos machinery (i.e., ATP synthase, adenine nucleotide transporter, inorganic phosphate transporter) are categorized as OPIs.

Compounds to be assessed for mitochondrial toxicity are provided to the cells at a designated time prior to the assay, then OCR is measured under basal (Basal OCR), oligomycin-induced (Oligo OCR), and FCCP-induced (FCCP OCR) conditions. Two control groups, in which the cells are treated with vehicle or Rot/AA, are required (Figure 2A) to provide positive and negative control values. FCCP and Rot/AA are also used as the reference compounds for detection of uncoupling and inhibition. Use of these mitochondrial modulators allow both the type and the magnitude of toxicity to be determined, as described in the next sections.

Inhibition (decrease in FCCP OCR): If treatment with a compound results in decreased FCCP OCR, then this is defined as inhibition (Figure 2A). Treatment of cells with an optimal concentration of FCCP will typically increase OCR to maximal rates. Under these conditions of higher substrate demand, the sensitivity of mitochondria to toxic compounds increases. Thus, if a compound treatment results in a decrease in FCCP OCR, compared to the vehicle group, this type of mitochondrial toxicity is defined as inhibition. Inhibition can be mediated by exert effects on transport, TCA cycle, FAO, ETC, or other upstream process (Figure 1), in which some component of mitochondrial function as been disrupted or inhibited (Figures 1 and 2B). Note that treatment of the cells with FCCP provides the specificity required to identify inhibitors, and increases the dynamic range for detection of inhibition compared to measurement of only Basal OCR.

Uncoupling (increase in Oligo OCR): If treatment with a compound results in increased Oligo OCR, then this is defined as uncoupling. Oligomycin, an inhibitor of the mitochondrial ATP synthase, will significantly reduce electron flow through the electron transport chain, resulting in a decrease of OCR, as observed with the vehicle upon injection of oligomycin (Figure 2A). If a compound treatment results in increased Oligo OCR compared to vehicle, this type of mitochondrial toxicity is defined as uncoupling, in which the ETC has been uncoupled from the oxidative phosphorylation machinery, i.e. ATP synthase, adenine nucleotide transporter (ANT), and inorganic phosphate transporter (P_i T). This effect is usually through protonophore action of the compound, shuttling protons across the mitochondrial inner membrane from the intermembrane space (IMS) to the matrix.

The result is that the mitochondria expend energy to re-establish a proton motive force by pumping protons from the matrix to the IMS, which causes an increase in respiration rate (OCR). However, mitochondrial ATP synthesis is usually compromised or altogether abrogated, ultimately having a deleterious effect on cell metabolism. Similar to measuring FCCP OCR, measuring Oligo OCR provides the specificity required to identify uncouplers, as well as increasing the dynamic range for detection of mitochondrial toxicity due to uncoupling, when compared to measurement of only Basal OCR.

OxPhos inhibition (decrease in only Basal OCR): Suppression of OxPhos activities (i.e. ATP synthase, ANT, and P_iT) is categorized as OxPhos inhibition (Figure 2B). While changes in Basal OCR can be used to assess mitochondrial toxicity, there are several drawbacks to inferring mitochondrial toxicity from only Basal OCR measurements. As noted above, the dynamic range of Basal OCR is less significant than the ranges of Oligo OCR or FCCP OCR. This results in lower sensitivity and often a decrease in signal-to-noise ratio. This problem is compounded by the fact that cells are often less sensitive to toxic compounds under basal respiration conditions, as the energetic demands of the cell are less. Nevertheless, Basal OCR is still informative for indicating OxPhos inhibition when only Basal OCR is inhibited significantly by a compound with neither Oligo OCR nor FCCP OCR decrease. Once a compound is identified as an OPI in this definition (Figure 2A), then further investigation is encouraged to validate the mode of toxicity. Details of the criteria used for determination of OPI are discussed further below.

In summary, based on responses in either FCCP OCR, Oligo OCR and/or Basal OCR of the test compounds compared to appropriate controls, the XF Mito Tox assay can identify three distinct types of mitochondrial toxicity: a) direct/indirect inhibition of the ETC or other mitochondrial processes, b) uncoupling of the ETC from OxPhos, and c) specific inhibition of the OxPhos machinery or any potential inhibition detected only by Basal OCR. Further, measuring OCR under bioenergetic conditions beyond basal respiration (i.e., Oligo OCR and FCCP OCR) allows for both increased specificity and sensitivity in the assessment of compounds for mitochondrial toxic effects.

Defining the mitochondrial toxicity index (MTI)

Having established an assay design that provides specificity (type of toxicity) and increased sensitivity, the next step was to devise a standardized scale on which the magnitude of toxicity is quantitated. This requires the inclusion of proper control measurements/groups in the assay to define the upper and lower boundaries of uncoupling and inhibition (i.e. to define the dynamic ranges of detecting uncoupling and inhibition). This dimensionless metric is designated as the mitochondrial toxicity index (MTI) and is derived and defined as follows.

Defining MTI for inhibition: Mitochondrial toxicity due to inhibition is defined as a decrease in FCCP OCR of the test compound compared to the negative control measurement (i.e. 0% inhibition), which in this case is the maximal FCCP OCR of the vehicle group. Conversely, 100% inhibition is defined by the positive control measurement, which is the minimal FCCP OCR of the Rot/AA group.

Derivation of the MTI value for inhibition takes the form:

$$\text{Inhibition MTI} = \frac{X - \text{NC}}{\text{NC} - \text{PC}}$$

Where:

X is the maximal FCCP OCR of the test compound.

NC is the negative control (maximal FCCP OCR of vehicle).

PC is the positive control (minimal FCCP OCR of Rot/AA group).

This can be expressed as:

$$\text{Inhibition MTI} = \frac{\text{Max FCCP OCR}_{\text{Test}} - \text{Max FCCP OCR}_{\text{Vehicle}}}{\text{Max FCCP OCR}_{\text{Vehicle}} - \text{Min FCCP OCR}_{\text{Rot/AA}}}$$

Applying this equation results in a negative fractional MTI value for inhibition, typically between 0 and -1, and is illustrated and described in Figure 3.

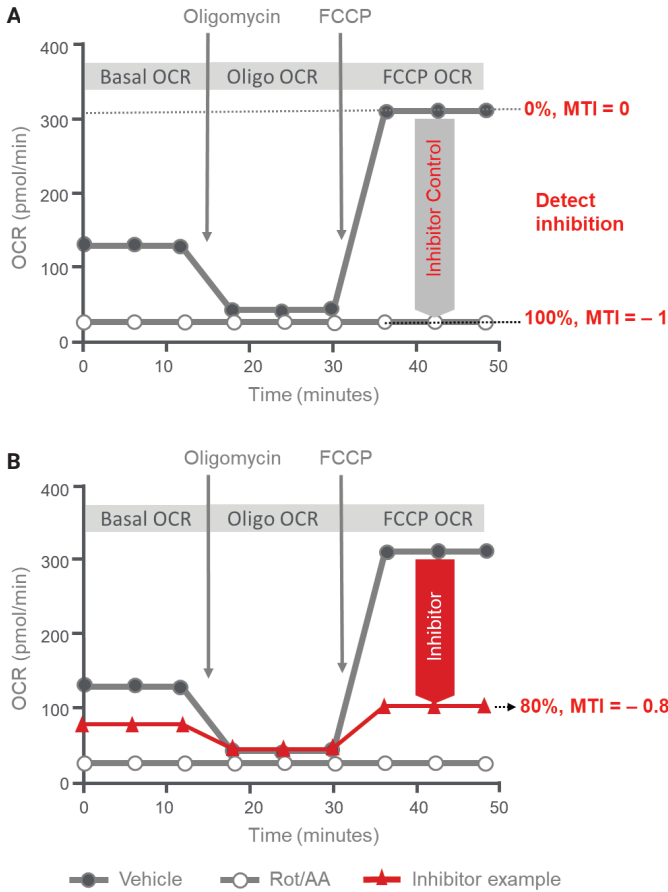


Figure 3. Defining the MTI for inhibition. (A) If a test compound results in a decrease in FCCP OCR, compared to that of the vehicle group (MTI = 0), then the compound is categorized as an inhibitor. Note that FCCP OCR of the Rot/AA group serves as a positive control for inhibition (MTI = -1). (B) Example of an inhibitor with MTI = -0.8.

Defining MTI for uncoupling: Mitochondrial toxicity due to uncoupling is defined and detected as an increase in Oligo OCR of the test compound compared to the negative control measurement (i.e., 0% uncoupling), which in this case is the minimal Oligo OCR of the vehicle group. Conversely, 100% uncoupling is defined by the positive control measurement, which is the maximal FCCP OCR of the vehicle group.

Derivation of the MTI value for uncoupling takes the form:

$$\text{Uncoupling MTI} = \frac{X - \text{NC}}{\text{PC} - \text{NC}}$$

Where:

X is the maximal Oligo OCR of the test compound.

NC is the negative control (minimal Oligo OCR of vehicle group).

PC is the positive control (maximal FCCP OCR of vehicle group).

This can be expressed as:

$$\text{Uncoupling MTI} = \frac{\text{Max Oligo OCR}_{\text{Test}} - \text{Min Oligo OCR}_{\text{Vehicle}}}{\text{Max Oligo OCR}_{\text{Vehicle}} - \text{Min Oligo OCR}_{\text{Vehicle}}}$$

Applying this equation results in a positive fractional MTI value for uncoupling, typically between 0 and 1, and is illustrated and described in Figure 4.

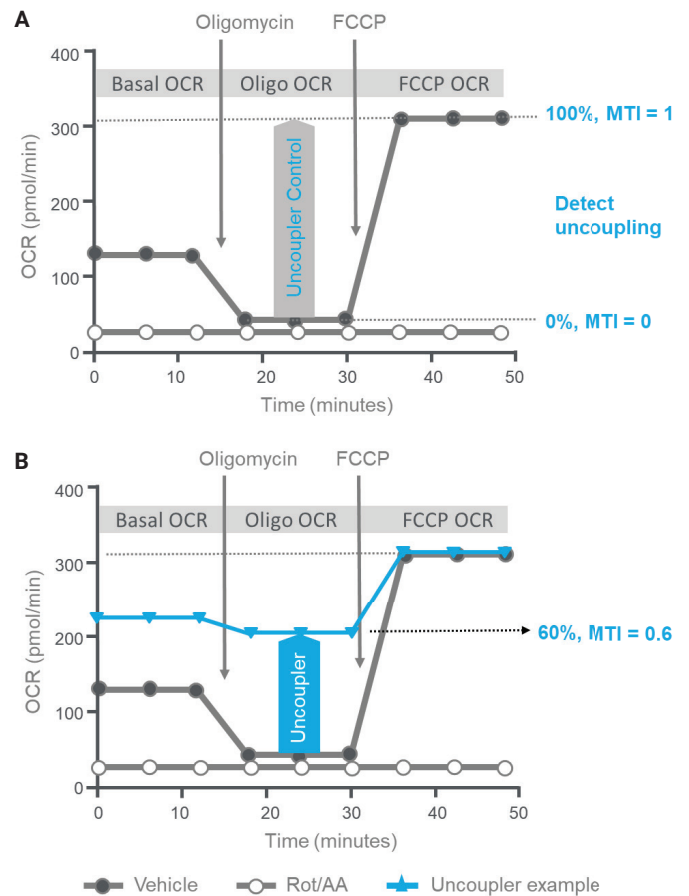


Figure 4. Defining the MTI. (A) If a test compound results in an increase in Oligo OCR, compared to that of the vehicle group (negative control, MTI = 0), then the compound is categorized as an uncoupler. Note that vehicle FCCP OCR serves as a positive control for uncoupling (MTI = 1). (B) Example of an uncoupler (nimesulide) with MTI = 0.6.

In summary, the MTI is the fraction value of test compound effect compared to respective controls for either uncoupling and/or inhibition. The MTI is calculated as positive index number for uncoupling and as negative index number for inhibition. The control groups and MTI scale is summarized in Table 1. Upon data transformation, both MTIs for uncoupling and inhibition are generated for each well. This single intuitive parameter facilitates the interpretation of the assay results by providing information for both the magnitude (absolute value) and type of mitochondrial toxicity (positive versus negative value).

Defining mitochondrial toxicity due to OPI: As noted above, a specific case of mitochondrial toxicity due to decreased mitochondrial function is the direct inhibition of the ATP synthase, or other components of the OxPhos machinery, which is defined as OPI. This type of inhibition typically results in a decrease in Basal OCR, while Oligo OCR and FCCP OCR are not significantly affected (Figure 5).

To discriminate OxPhos inhibition from other modes of toxicity, the following two criteria are applied. A test compound must satisfy both criteria to be designated as OPI (if criteria 1 and criteria 2):

Criteria 1: z-score for minimal Basal OCR < -3

Criteria 2: z-score for maximal FCCP OCR > -3

$$\text{Z-score for Basal OCR} = \frac{\chi - \mu}{\sigma}$$

Where:

χ is the Basal OCR of the test compound.

μ is the mean Basal OCR of vehicle group.

σ is the standard deviation of Basal OCR of vehicle group.

and

$$\text{Z-score for FCCP OCR} = \frac{\chi - \mu}{\sigma}$$

Where:

χ is the maximal FCCP OCR of the test compound.

μ is the mean of maximal FCCP OCR of the vehicle group.

σ is the standard deviation of maximal FCCP OCR of the vehicle group.

Table 1. Control measurements and groups used for MTI scale definition for determining uncoupling and inhibition.

Mode	Control	Measurement	Group	Uncoupling (%)	Inhibition (%)	MTI
Uncoupling	Negative	Minimal Oligo OCR	Vehicle	0	NA	0
	Positive	Maximal FCCP OCR	Vehicle	100	NA	1
Inhibition	Negative	Maximal FCCP OCR	Vehicle	NA	0	0
	Positive	Minimal FCCP OCR	Rot/AA	NA	100	-1

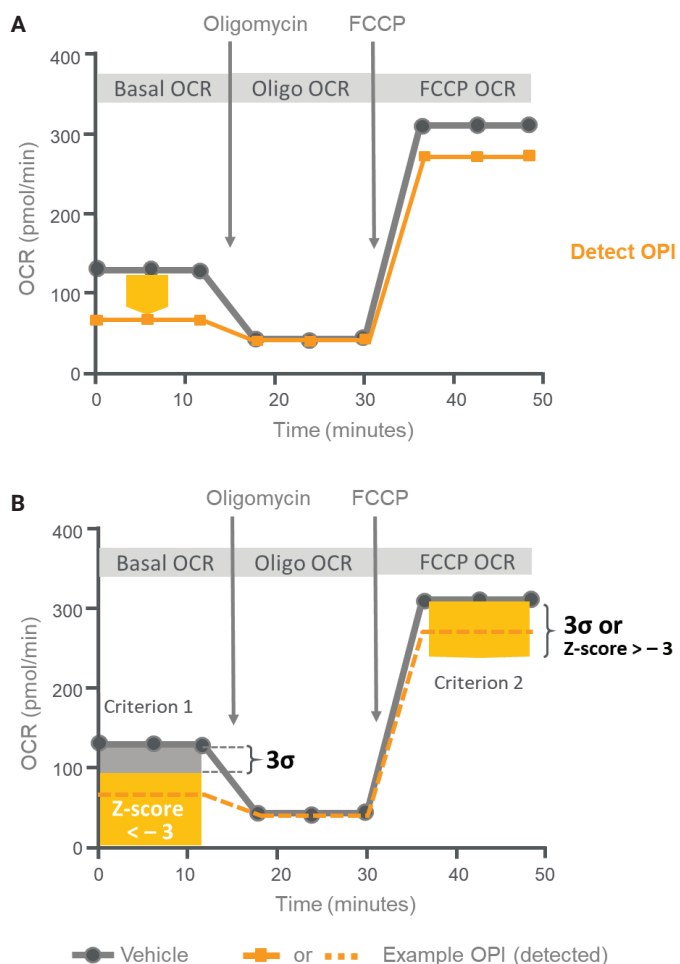


Figure 5. Defining conditions or criteria for OPIs. (A) Kinetic graph illustrating definition of OxPhos inhibition. (B) Two criteria used for detection of OPI. If a test compound treatment results in a decrease in Basal OCR (z-score < -3) and does not result in a significant decrease in FCCP OCR (z-score > -3), then the compound is categorized as an OPI. Note that no MTI value is calculated for OPIs.

Using the z-score enables for specific discrimination ($p < 0.005$) of potential OxPhos inhibition events (versus uncoupling or inhibition) by correlating the window (criteria) of OPI detection to the standard error (i.e. noise) obtained for Basal OCR and FCCP OCR of the vehicle group. These criteria are used to decrease the chances of reporting false positives for this mode of toxicity. Compounds are detected as OPIs for mitochondrial toxicity if these criteria are met. Upon observing detection of OPI, it is encouraged to perform downstream assays (e.g. XF Mito Tox dose-response assay or other orthogonal assays) to further investigate and characterize this type of toxicity.

XF Mito Tox assay performance metrics

Having defined a quantitative metric of mitochondrial toxicity (MTI value), there is also the requirement for the evaluation of the quality of assay performance. The Z' calculated from OCR measurements for the control groups (Table 1) is used to assess the assay performance and ensure robust MTI output. Z' , a screening window coefficient can be used as a dimensionless statistical characteristic for high-throughput screening when two extreme reference controls over the dynamic range of the measurement are used.⁵ Figure 6A illustrates the assay windows where the Z' 's are assessed. These assay windows are used calculate MTIs for inhibitor and uncoupler.

In the XF Mito Tox assay, corresponding Z' for uncoupling and inhibition are calculated as follows:

$$Z' = 1 - \frac{(3\sigma_{PC} + 3\sigma_{NC})}{|\mu_{PC} + \mu_{NC}|}$$

Where:

μ_{PC} is the mean OCR of positive control.

μ_{NC} is the mean OCR of negative control.

σ_{PC} is the standard deviation of positive control.

σ_{NC} is the standard deviation of negative control.

To validate the MTI-based toxicity screening and to test the robustness and consistency of the assay, Z' across plates/days were compared by using HepG2 cells, following the standard XF Mito Tox assay protocol described in the Agilent Seahorse XF Mito Tox Assay Kit User Guide.⁶ As shown in Figure 6, XF Pro M plates are divided into two groups, vehicle and Rot/AA, using two reciprocal plate maps (Figure 6B). In general, it is desired to achieve Z' of greater than 0.5 to be confident of screening assay results. The results show, for both normalized and not normalized data, Z' values of >0.5 for uncoupling and inhibition are consistently

obtainable and are independent of plate layout, from plate to plate or day to day. (Figure 6C). The data demonstrate that the dynamic assay window of Seahorse XF Mito Tox assay using HepG2 cells is valid for mitochondrial toxicity screening application.

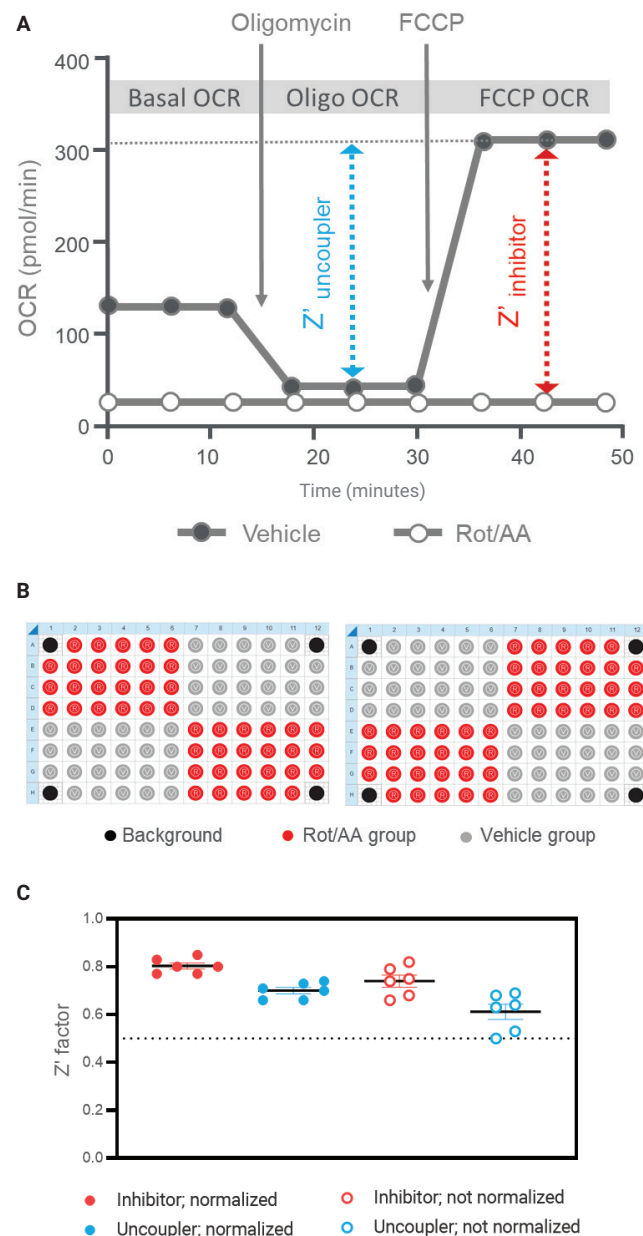


Figure 6. Assessment of Agilent Seahorse XF Mito Tox assay performance using Z' . (A) Dynamic ranges used for calculating Z' for uncoupling (blue) and inhibition (red). (B) Plate map showing the layout of control groups ($n = 46$ per plate); vehicle (gray) and Rot/AA (red). (C) Summary of Z' obtained over three consecutive days with or without data normalized to cell numbers counted by the Agilent Seahorse XF Imaging and Normalization System.

Applications

The basic applications of the XF Mito Tox assay include screening of compounds at a fixed concentration and dose-response analysis with calculation of IC_{50} or EC_{50} values. Proof-of-concept examples of these two applications using a small library of compounds known to elicit mitochondrial toxicity are provided. All assays were performed using the protocol described in the Agilent Seahorse XF Mito Tox Assay Kit User Guide⁶, and the data were processed and analyzed using Agilent Seahorse Analytics.

Screening analysis

As an application example of the assay, several compounds known to elicit different mitochondrial toxicity effects (including uncoupling, direct ETC inhibition, and direct OxPhos inhibition) were evaluated with the XF Mito Tox assay. HepG2 cells were exposed to the eight compounds at a fixed concentration (100 μ M) for 1 hour, then the XF Mito Tox assay was performed. The OCR measurements were transformed to MTI values for each compound using Seahorse Analytics which are presented in the bar chart and plate map views (Figure 7). In this study, the type of toxic effect was identified (inhibition, uncoupling, or OxPhos inhibition) and quantitated via the MTI values. The results are in good agreement with previous reports.⁷⁻¹⁰ For detailed discussion of results, see the Agilent application note by Kam *et al.*¹¹

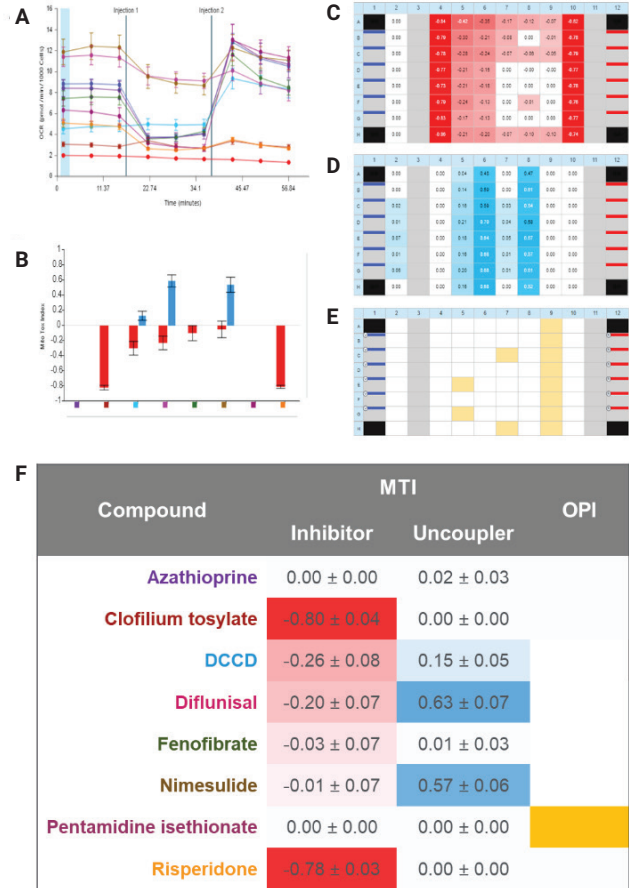


Figure 7. Results of an Agilent Seahorse XF Mito Tox screening assay. (A) XF kinetic OCR data showing test compounds and control groups. (B) Bar chart of resulting MTI values for each compound. (C to E) Plate heat map views displaying inhibitor and uncoupler MTIs, and OPI detection. (F) Summary table of MTI values and OPI detection for each test compound. (n = 8).

Dose-response analysis

Another key consideration for this methodology is the effect of compound concentration on the magnitude of mitochondrial toxicity. Because MTI values are calculated for each well, this parameter can also be used to calculate dose-response curves to assess the magnitude of toxicity with respect to compound concentration, i.e., the MTI value versus compound dose. In Seahorse Analytics, EC_{50} and IC_{50} values are calculated using the four-parameter dose-response curve fitting models based on the Hill equation.¹²

EC_{50} where Hill slope >0 ,

$$MTI = Bottom + \frac{Top - Bottom}{1 + \left(\frac{EC_{50}}{Dose}\right)^{Hill\ slope}}$$

IC_{50} where Hill slope <0 ,

$$MTI = Bottom + \frac{Top - Bottom}{1 + \left(\frac{IC_{50}}{Dose}\right)^{Hill\ slope}}$$

To demonstrate the utility of the XF Mito Tox assay in assessing the potency of mitochondrial toxic compounds, a dose-response assay was performed using two inhibitors (clofilium tosylate and risperidone) and two uncouplers (diflunisal and nimesulide). HepG2 cells were exposed to these compounds for 1 hour at 10 concentrations prior to performing the assay. As shown in Figure 8, the kinetic OCR measurements were translated into MTI-based dose-response curves to obtain IC_{50} and EC_{50} values for inhibition and uncoupling, respectively.

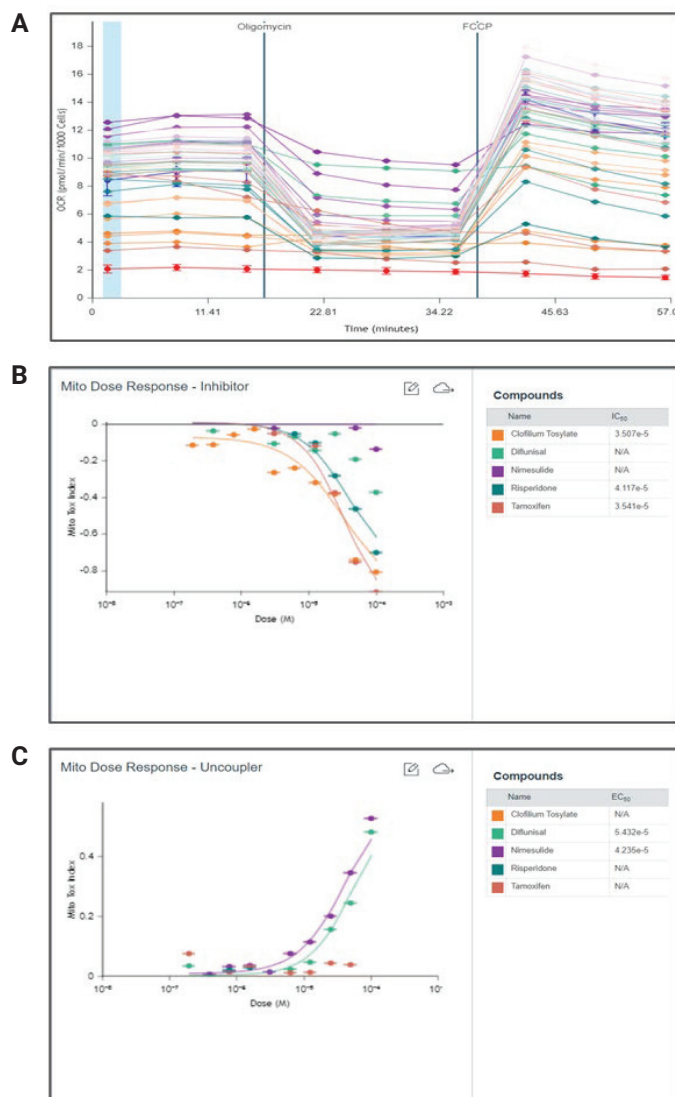


Figure 8. Dose-response analysis of mitochondrial toxic compounds. (A) Kinetic OCR measurements. (B) MTI values versus dose of inhibitors clofilium tosylate and risperidone with respective IC_{50} values. (C) MTI values versus dose of uncouplers diflunisal and nimesulide with respective EC_{50} values.

Effects of extended compound treatment: mitochondrial toxicity versus cytotoxicity and normalization of data:

In addition to the effects of concentration on mitochondrial toxicity, another key variable to consider is the length of time of exposure of the compound to the cells. The XF Mito Tox assay is primarily designed to assess acute mitochondrial toxicity, i.e. detect deleterious effects on mitochondrial function within minutes to hours. While longer compound exposure times are often warranted (and even necessary, depending on kinetics of compound uptake by the cells), these longer-term compound exposures (hours to days) may have downstream effects due to either mitochondrial dysfunction expediting cytotoxicity, or conversely, cytotoxicity expediting mitochondrial dysfunction. Note that in certain cases, data normalization to cell numbers may mask cytotoxic effects. As illustrated in Figure 10, the MTI value does not change, but the cell number is decreased during the 48 hours of exposure to 500 μM nimesulide. The normalized data suggest MTI values for uncoupling and inhibition do not change significantly over time (4, 24, and 48 hours). However, if the data are not normalized, the magnitudes of uncoupling and inhibition are decreased and increased, respectively, at longer time points (4 versus 24 and 48 hours). These changes in MTI values can be attributed to cytotoxic effects of nimesulide at 24 and 48 hour time points, which are evident by the significant decrease in cell number (63 and 34% of control, respectively) at these longer times of compound exposure. In summary, it is encouraged to carefully inspect resulting data to ensure accurate interpretation.

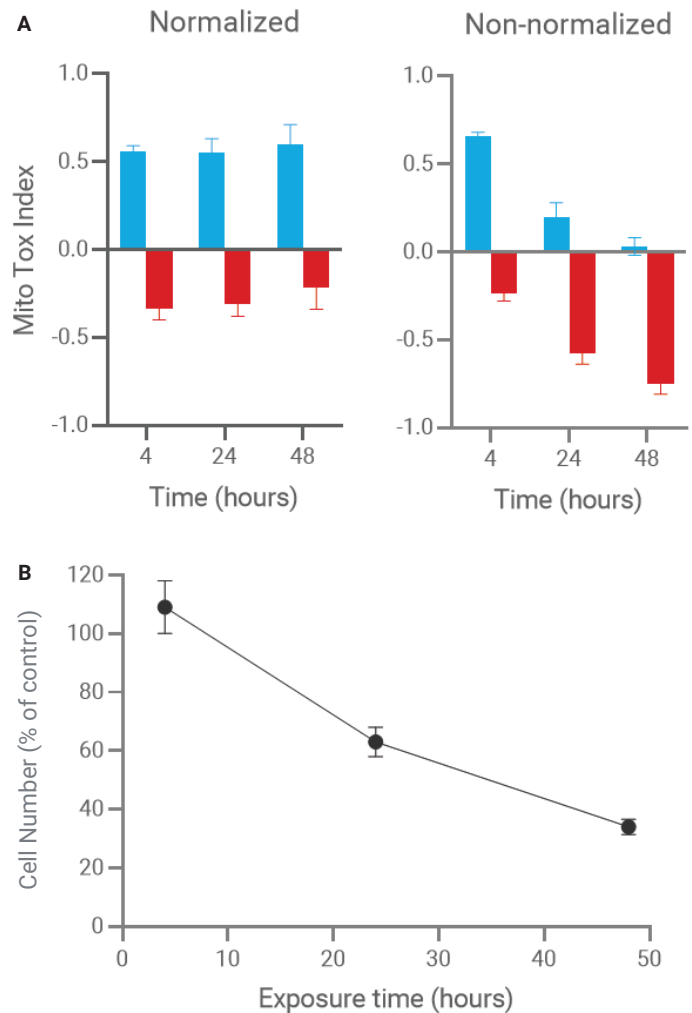


Figure 10. Effects of cytotoxicity and data normalization on MTI values: (A) MTI values for 500 μM nimesulide at 4, 24, and 48 hours post compound exposure when using normalized and non-normalized OCR data to calculate MTI values. (B) Cell number (% of control) at 4, 24, and 48 hours post compound exposure.

Conclusion

In summary, direct measurement of mitochondrial oxygen consumption is a specific and sensitive indicator to assess drug-induced mitochondrial toxicity, a common issue in therapeutic development. This white paper introduces the Agilent Seahorse XF Mito Tox assay, a customized assay, which is used to assess mitochondrial toxicity, and has the ability to identify and specifically distinguish among different modes of mitochondrial toxicity with high sensitivity. Further, this assay introduces a standardized quantitative measurement of the magnitude of toxicity: the MTI. In addition, to provide assurance of resulting data quality, a metric of assay performance is provided (Z'). This assay design and respective output parameters enable both rapid, straightforward implementation and intuitive, confident data interpretation for either screening or dose-response types of assays when examining mitochondrial toxicity of therapeutic compounds.

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