Application Note

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A Customized XF Workflow for Detection and Characterization of Mitochondrial Toxicity

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Abstract

A customized XF solution that allows detection and quantitative assessment of compounds eliciting mitochondrial toxicity has been developed. The Agilent Seahorse XF Mito Tox assay provides:

- 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 setup for either compound screening or dose-response analysis

This application note provides proof of concept and examples for compound screening and dose-response experiments using the XF Mito Tox assay and interpretation of resulting data using the Mito Tox index (MTI). Discussion of strategic assay design, performance metrics, and specific cases when using the XF Mito Tox assay are also presented.

Agilent Seahorse XF technology is a label-free, integrated platform that seamlessly combines the Agilent Seahorse XF Pro analyzer, sensor cartridges, assay kits, and software to deliver functional cellular metabolic data with real-time kinetics. The XF Mito Tox assay contains validated reagents and features a streamlined workflow supported by Agilent Wave Pro software and Agilent Seahorse Analytics tools that provide intuitive assay design, performance, data-quality assessment, and data interpretation. Altogether, this standardized XF solution allows users to obtain robust results and actionable conclusions quickly, and reduce assay development needs for assessment of mitochondrial toxicity.

Introduction

Mitochondria perform critical functions in the regulation of cellular physiological mechanisms, including ATP generation, metabolic control, immune response, signal transduction, and apoptosis.[1] Mitochondrial damage involves processes or insults that result in dysfunctional mitochondria, whether by direct enzymatic inhibition, oxidative damage, mutation of mitochondrial/nuclear DNA, and/or other upstream cellular processes.

Mitochondria have also been implicated as unintended targets of pharmaceutical and therapeutic compounds, resulting in disruption to mitochondrial function (Figure 1). This drug-induced mitochondrial toxicity may result in brain, cardiac, liver, muscle, and/or kidney injury: organs that rely heavily on oxidative phosphorylation or serve as the primary organ of drug metabolism.[2] Mitochondrial toxicity has been described for many different drug classes. Many agricultural, industrial, and pharmaceutical chemicals that are classified as harmful to human and environmental health exert their effects through the mechanism of mitochondrial toxicity.[3]

Therefore, sensitive, specific, and accurate detection of mitochondrial toxicity is a key consideration during the development of therapeutic compounds to decrease both drug-candidate attrition and postmarket drug withdrawals. Of many applicable preclinical organelle-based and *in vitro* cell models, oxygen consumption-based detection methods have been described as the most informative and specific among assays used to assess mitochondrial dysfunction and toxicity.[4-6] Using Seahorse XF technology to provide a direct assessment of mitochondrial function through measurement of oxygen consumption has been well documented as a specific and sensitive marker/indicator of mitochondrial toxicity.[4,7]

This application note introduces a customized XF assay for simplified detection and evaluation of mitochondrial toxicity. Using the Seahorse XF Mito Tox assay kit in conjunction with the Seahorse XF Pro analyzer and dedicated software features enables streamlined, sensitive detection and characterization of mitochondrial toxicants. This assay allows discrimination among different modes of mitochondrial toxicity, delivers a standardized quantitative parameter of the magnitude of the toxicity, the MTI, and provides a metric for the evaluation of assay quality and performance. This solution, which is automation-compatible, is designed to provide rapid, straightforward assay design and intuitive data interpretation for either compound screening or dose-response types of assays. The solution is designed to decrease the time and effort in detection and assessment of mitochondrial toxicants.

A customized XF assay design for standardized mitochondrial toxicity assessment

Drugs can disrupt mitochondrial function in many direct or indirect 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, or by uncoupling the ETC from ATP synthesis.[7] This drug-induced mitochondrial dysfunction typically causes perturbations in the mitochondrial oxygen consumption rate (OCR), which can be assessed directly by Seahorse XF technology.

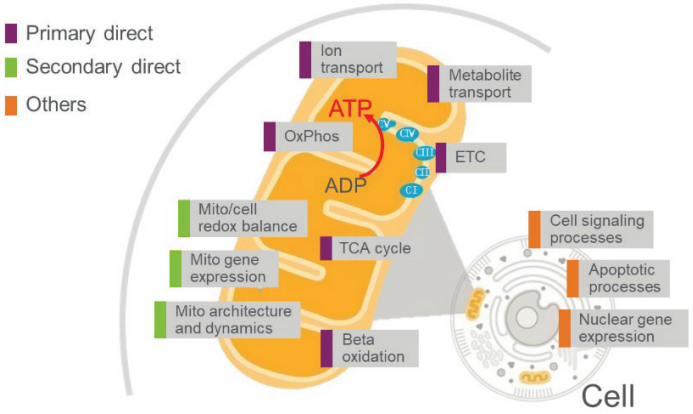


Figure 1. Key mitochondrial and cellular processes that are potential targets of drug-induced mitochondrial toxicity. Primary direct targets include metabolite and ion transport, tricarboxylic acid cycle (TCA), fatty acid oxidation (FAO), electron transport chain (ETC) and oxidative phosphorylation (OxPhos) 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. Note that this figure is meant for illustrative purposes and is not an exhaustive list.

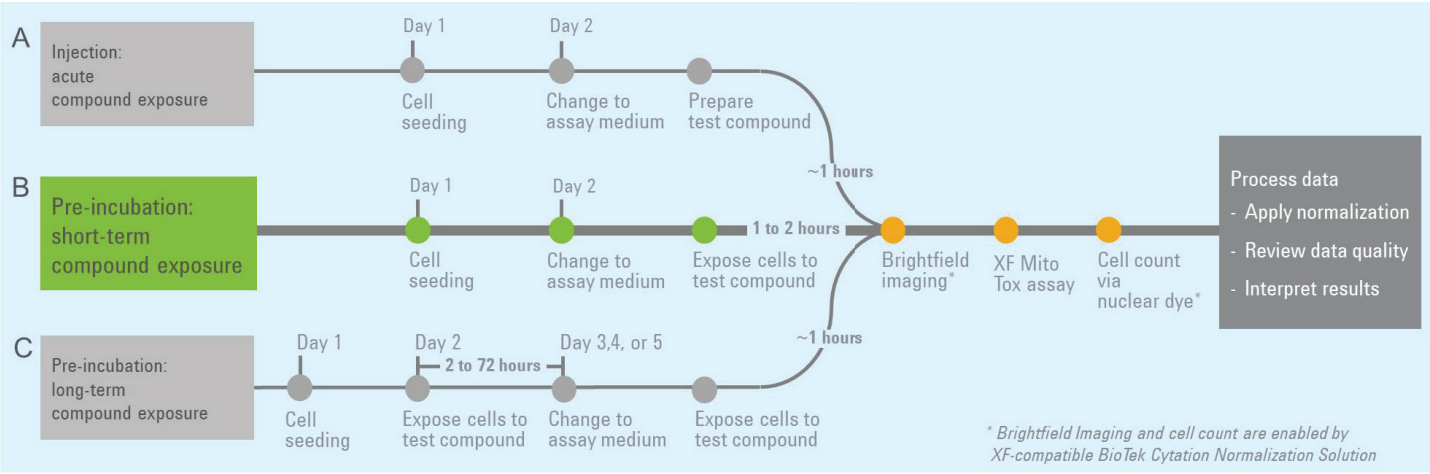


Figure 2. Streamlined workflow for the Agilent Seahorse XF Mito Tox assay to investigate acute, short-term, and long-term effects of compounds. Note that post assay cell counting for data normalization is recommended but optional.

In the Seahorse XF Mito Tox assay, compounds to be assessed for mitochondrial toxicity are provided to the cells at a designated time before the assay (Figure 2), then OCRs are measured in real time under basal (Basal OCR), oligomycin-induced (Oligo OCR), and FCCP-induced (FCCP OCR) conditions (Figure 3A).

Based on responses in Basal OCR, Oligo OCR, and/or FCCP OCR of the test compounds compared to appropriate controls, the XF Mito Tox assay can identify three distinct types of mitochondrial toxicity:

- Direct/indirect inhibition of the ETC or other mitochondrial processes
- Uncoupling of the ETC from OxPhos
- Other inhibition potentially targeting the OxPhos machinery components, i.e. complex V, adenine nucleotide transporter (ANT), and the inorganic phosphate transporter (P_iT) (Figure 3B).

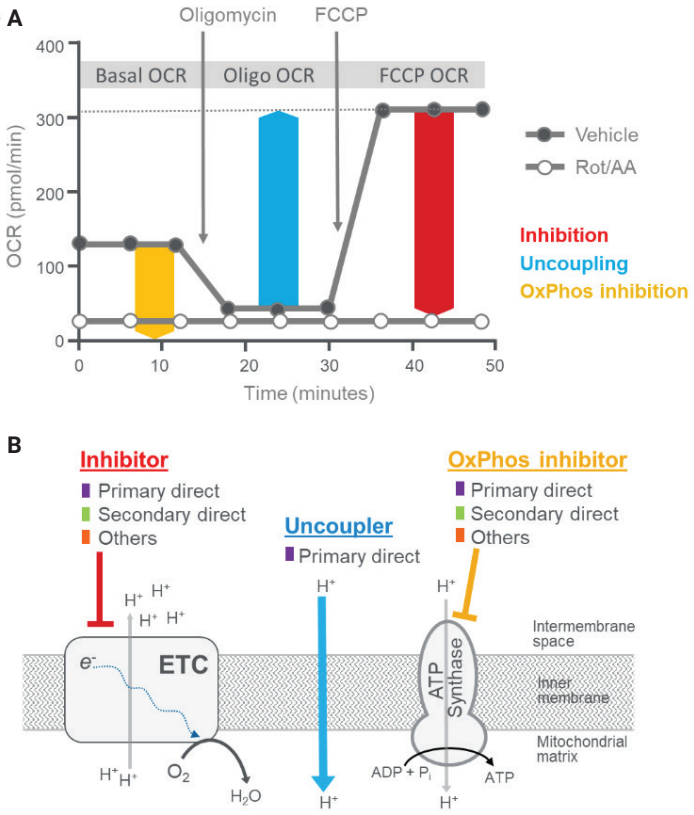


Figure 3. Modes of mitochondrial toxicity identified by the Agilent Seahorse XF Mito Tox assay. (A) Assay kinetic graph illustrating control groups (vehicle and Rot/AA) with oligomycin and FCCP injections. The effect of inhibitors, uncouplers, and OxPhos inhibitors (OPIs) are detected by measuring changes 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, ANT, P_iT) are categorized as OPIs.

To discriminate among the modes of mitochondrial toxicity described above, as well as to quantitate the magnitude of toxicity, the MTI value was derived. Mitochondrial toxicity due to inhibition, where inhibition is defined and detected as a decrease in FCCP OCR by the test compound compared to maximal FCCP OCR of the vehicle group, results in a negative MTI value (typically between 0 and –1) and is illustrated and described in Figure 4A. Note that Rot/AA OCR serves as a positive control for inhibition (100% inhibition, MTI = –1), while vehicle FCCP OCR serves a negative control (0% inhibition, MTI = 0). In addition, positive MTI values for inhibition are reported as 0.

Mitochondrial toxicity due to uncoupling, where uncoupling is defined and detected as an increase in Oligo OCR of the test compound compared to minimal Oligo OCR of the vehicle group, results in a positive MTI value (typically between 0 and 1) and is illustrated and described in Figure 4B. Note that vehicle FCCP OCR serves as a positive control for uncoupling (100% uncoupling, MTI = 1), while vehicle Oligo OCR serves as a negative control (0% uncoupling, MTI = 0). In addition, negative MTI values for uncoupling are reported as 0.

A particular case of mitochondrial toxicity due to decreased mitochondrial function is the potential inhibition of the ATP synthase, or other components of the OxPhos machinery (e.g., ANT or P_iT). This type of inhibition often shows a significant decrease in Basal OCR, while Oligo OCR and FCCP OCR are not significantly affected (Figure 4C). In these cases, test compounds will be flagged to signal for mitochondrial toxicity due to potential inhibition of OxPhos components (OPIs). If warranted, downstream experiments (e.g., dose-response assay, Agilent Seahorse XF Cell Mito Stress Test, Agilent Seahorse XF Substrate Oxidation Stress Test and/or orthogonal assays) may be performed to further investigate the toxic effects/mechanism of the compound. Note that no MTI value will be calculated for this specific type of inhibition.

Full details of the MTI derivation, calculations, and XF data transformation, as well as the OPI detection criteria and reporting can be found in the Agilent white paper by Rogers *et al.*[8]

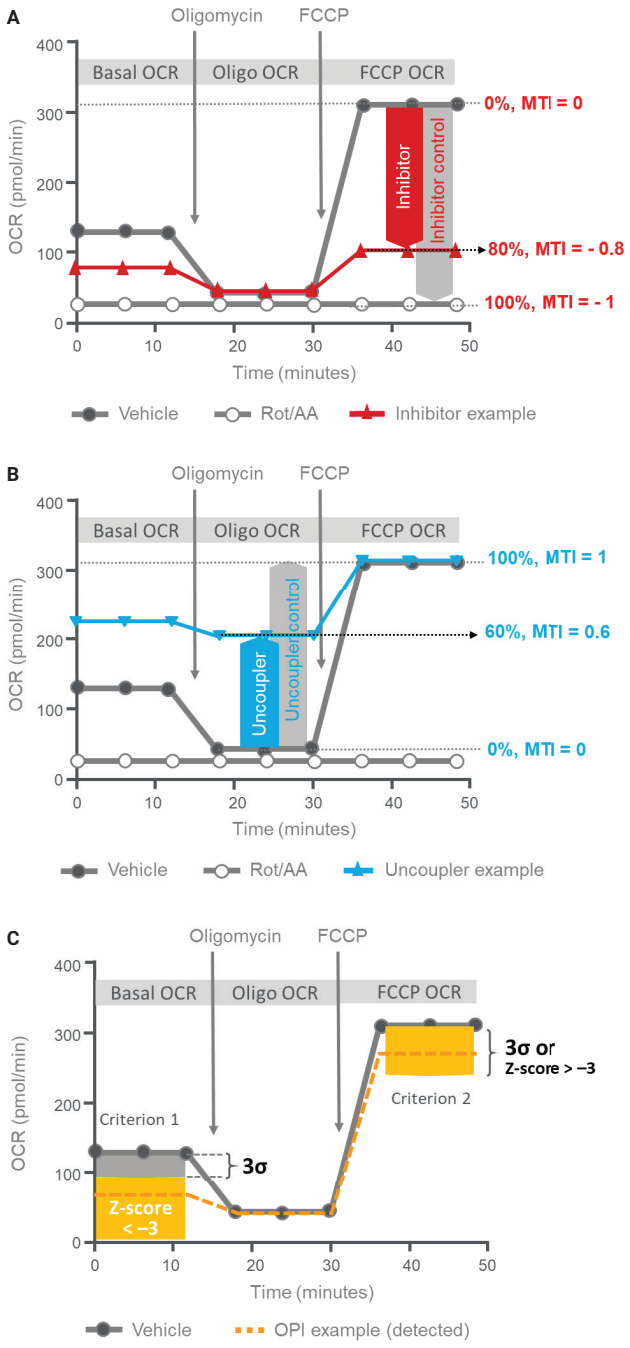


Figure 4. Agilent Seahorse XF Mito Tox assay kinetic OCR profiles illustrating the definition of MTI and criteria used to assess the mode and the magnitude of mitochondrial toxicity. (A) MTI definition for inhibition; the red line illustrates example kinetic OCR trace of a compound showing mitochondrial toxicity through inhibition, with an MTI = –0.8. (B) MTI definition for uncoupling; the blue line illustrates example kinetic OCR trace of a compound showing mitochondrial toxicity through uncoupling, with an MTI = 0.6. (C) Detecting inhibition of OxPhos machinery. Two criteria are required: z-score for Basal OCR <–3 and z-score for FCCP OCR >–3. Note that MTI values are not calculated for OPIs. The yellow line illustrates an example kinetic OCR trace of a compound detected for mitochondrial toxicity through OxPhos inhibition.

In summary, based on responses in 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:

- Direct/indirect inhibition of the ETC or other mitochondrial processes
- Uncoupling of the ETC from OxPhos
- Potential inhibition of the OxPhos machinery

Further, measuring OCR under bioenergetic conditions beyond basal respiration (i.e., Oligo OCR and FCCP OCR) allows both increased specificity and sensitivity in the assessment of compounds for mitochondrial toxic effects.

Experimental

XF assays

HepG2 cells were seeded in Agilent Seahorse XF Pro M cell culture microplates at a density of 2.0×10^4 cells per well and cultured in low-glucose DMEM (Gibco 11885) supplemented with 2 mM GlutaMAX and 10% serum. Plates were kept in the thermostat cabinet for 1 hour after seeding, then incubated at 5% CO_2 at 37 °C in a humidified atmosphere for 24 hours. On the following day, cells were washed twice with XF assay medium (Agilent Seahorse XF DMEM medium, pH 7.4, supplemented with 10 mM Agilent Seahorse XF glucose solution, 1 mM Agilent Seahorse XF pyruvate solution, and 2 mM Agilent Seahorse XF glutamine solution) and incubated at 37 °C, without CO_2 for 60 minutes. Compound solutions as well as rotenone/antimycin solution (final concentration of 0.5 µM) were added at the time of cell washing. Cell plates were then transferred to the XF Pro analyzer for XF Mito Tox assay performance, using sequential injection of oligomycin (1.5 µM final), FCCP (1.5 µM final). After the XF assay, cell plates were imaged using Agilent Seahorse XF Imaging and Normalization system with an Agilent BioTek Cytation 5 cell imaging multimode reader to determine the cell numbers in each well, which were used to normalize assay measurements.

All XF assays were performed following the procedures described in the Agilent Seahorse XF Mito Tox Assay Kit user guide[9], including preparation of compounds, reagents, and sensor cartridges.

Automation with the Bravo automated liquid handling platform The Agilent Bravo automated liquid handling platform was used to streamline XF assay preparation. For all assays, cell washing was performed using the Bravo platform, leaving a final volume of 100 µL per well in preparation for addition of 100 µL of 2x compound solutions. For titration assays and the Enzo library screen, the Bravo platform was used to prepare and transfer compound solutions to the cell plates following plate washing.

The labware used is described in the Bravo Seahorse Assay Workbench user guide.[10] The cell-washing protocol is a modification of the protocol included with the Bravo Workflow for Seahorse XFe96 Sample Preparation.[11] Serial dilutions and compound library dilutions were performed in a 96-well reservoir to allow transfer of pretreatment solution in a single step. Injection ports were loaded manually, but this step may also be performed using the Bravo platform if desired.

Table 1. Assay steps carried out manually or with the Agilent Bravo platform.

	Z' Determination	Ten-Compound Screen	Ten-Step, Two-Fold Serial Dilution	Eighty-Compound Screen
Cell Washing	Bravo platform	Bravo platform	Bravo platform	Bravo platform
2x Compound Prep	Manual	Manual	Bravo platform	Bravo platform
2x Compound Transfer	Manual	Manual	Bravo platform	Bravo platform

XF Mito Tox assay data analysis and interpretation

Analysis and interpretation of XF Mito Tox assay result files were performed using Seahorse Analytics, a Web-based software platform. Using kinetic OCR measurements, dedicated tools (widgets) in Seahorse Analytics automatically calculate key Mito Tox assay parameters, including MTI values, IC_{50} and/or EC_{50} values, as well as an assay performance metric, Z' (Figure 5). These parameters are then presented as MTI heat maps, MTI bar charts, dose-response curves and Z' assessment. The assay design approach, derivation of MTI, and equations used for calculation of XF Mito Tox assay parameters are described in detail in the Agilent white paper by Rogers *et al.*[8] Instructions for analysis and interpretation of XF Mito Tox assay results using Seahorse Analytics are described in the XF Mito Tox Assay Kit User Guide.[9]

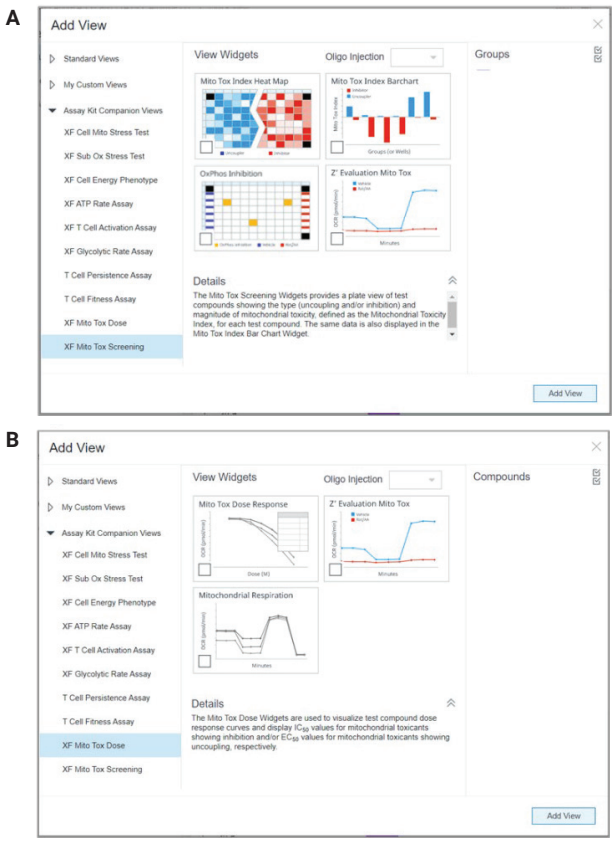


Figure 5. Agilent Seahorse Analytics Mito Tox assay companion views. (A) XF Mito Tox Screening widgets, including an MTI heat map and bar chart, an OPI detection heat map, and Z' evaluation of assay performance. (B) XF Mito Tox Dose widgets, including dose-response curves with IC_{50} and EC_{50} values, kinetic mitochondrial respiration (OCR) graphs, and Z' evaluation of assay performance.

Glu/Gal: total ATP assays

HepG2 cells (HB-8065, ATCC) were cultured in high-glucose DMEM (41965039, Gibco) for the "Glu" condition or glucose-free DMEM (11966025, Gibco) supplemented with 10 mM galactose (48359, Sigma-Aldrich) for the "Gal" condition. Both media were also supplemented with 10% FBS (F0804, Sigma-Aldrich), 1% penicillin/streptomycin (P4333, Sigma-Aldrich) and 1 mM Seahorse XF pyruvate solution (part number 103578-100). Cells were maintained at 5% CO_2 at 37 °C in a humidified atmosphere. Before the cells were seeded for the Glu/Gal assay, cells were allowed to adjust to the "Gal" condition for at least three passages.

Glu/Gal assays were performed and interpreted based on previously published protocols.[5,12] Cells grown in either Glu or Gal medium were seeded in growth medium onto 96-well Agilent cell culture microplates (part number 204626-100) at a density of 2×10^4 cells/well. Plates were kept at room temperature for 1 hour after plating, then incubated at 5% CO_2 at 37 °C in a humidified atmosphere for 24 hours. Cells were then treated with test compounds or vehicle in concentrations between 100 and 0.14 µM (1:3 dilution from 10 mM DMSO stock) by adding 1 µL of the diluted compound solution (1% DMSO in well for all) before returning the plates into the incubator for 24 hours. Plates were sealed with AeraSeal film (A9224, Merck Aldrich) during both incubation steps to avoid uneven evaporation. ATP level, as an indicator for cell viability, was assessed using a CellTiter-Glo Luminescent Cell Viability Assay (G7570, Promega) following the manufacturer's instructions. Luminescence was measured using either an Agilent BioTek Synergy H1 multimode reader or a Cytation 5 cell imaging multimode reader using the default luminescence detection protocol. Each condition was tested using n ≥2.

Cell viability was calculated relative to the vehicle control wells on the plate (100%) using Agilent BioTek Cytation 5 Gen5 software (version 11). IC_{50} values were calculated using a four-parameter logistic fit in GraphPad Prism 8. Bottom and top values were fixed to 0% and 100%, respectively. Fits with R^2 values <0.5 were discarded.

The ratio of the IC_{50}(Glu):IC_{50}(Gal) was calculated for each independent biological replicate and then averaged. Compounds showing an estimated IC_{50} >100 µM in Glu and Gal conditions were considered not active (NA).

Compound results were sorted into one of the following categories (Table 2).

Table 2. Compound result catagories.

Ratio of IC_{50}(Glu)/IC_{50}(Gal)	Relative Mitochondrial Toxic Effect
>>3 or >3	Strong mitochondrial toxic effect
2–3*	Mixed toxicity; mitochondrial toxicity contributes to toxicity of compound
<2	No or minimal mitochondrial component to the compound's effect in this assay

* If estimated IC_{50}(Glu) > 100 µM, a ratio >3 or >> 3 could potentially be observed, indicating a strong mitochondrial toxic effect of the compound

Materials

Materials	Material Vendor	Part Number
HepG2	ATCC	HB-8065
DMEM medium, low glucose	Gibco	11885
GlutaMAX	Gibco	35050061
Fetal bovin serum	HyClone	
Seahorse XF Pro M FluxPak	Agilent Technologies	103775-100 or 103777-100
Seahorse XF DMEM medium, pH 7.4	Agilent Technologies	103575-100
Seahorse XF 1.0 M glucose solution	Agilent Technologies	103577-100
Seahorse XF 100 mM pyruvate solution	Agilent Technologies	103578-100
Seahorse XF 200 mM glutamine solution	Agilent Technologies	103579-100
Seahorse XF Mito Tox assay kit	Agilent Technologies	103595-100
SCREEN-WELL Hepatotoxicity library	Enzo Life Sciences	
Test compounds	Sigma-Aldrich	Various
Seahorse XF Pro analyzer	Agilent Technologies	
Cytation 5	Agilent Technologies	

Results and discussion

Assay performance evaluation

Having defined a quantitative metric of mitochondrial toxicity (the MTI value), a demonstration of the use of the parameters for the evaluation of the quality of assay performance is conducted. Figure 6A illustrates the control groups and the assay windows where the Z' factors are assessed and MTIs are calculated for inhibitor and uncoupler, respectively. Z' across plates and from different days were compared using HepG2 cells exposed to vehicle or Rot/AA solutions. As shown, the XF Pro M plate was divided into two groups using two reciprocal plate maps (Figure 6B). The results show, for both normalized and not normalized data, Z' >0.5 for uncoupling and inhibition are consistently obtainable and are independent of changes in plate layout or plate-to-plate and day-to-day performance (Figure 6C). The data demonstrate that the dynamic assay windows for uncoupling and inhibition is valid for the assessment of mitochondrial toxicity screening applications using HepG2 cells. This strategy to assess Z' values is encouraged before performance of the XF Mito Tox assay with test compounds. In addition to initial assay validation, the Z' value may then be used to track assay-to-assay performance consistency. Further details of the Z' calculations can be found in the Agilent white paper by Rogers *et al.*[8]

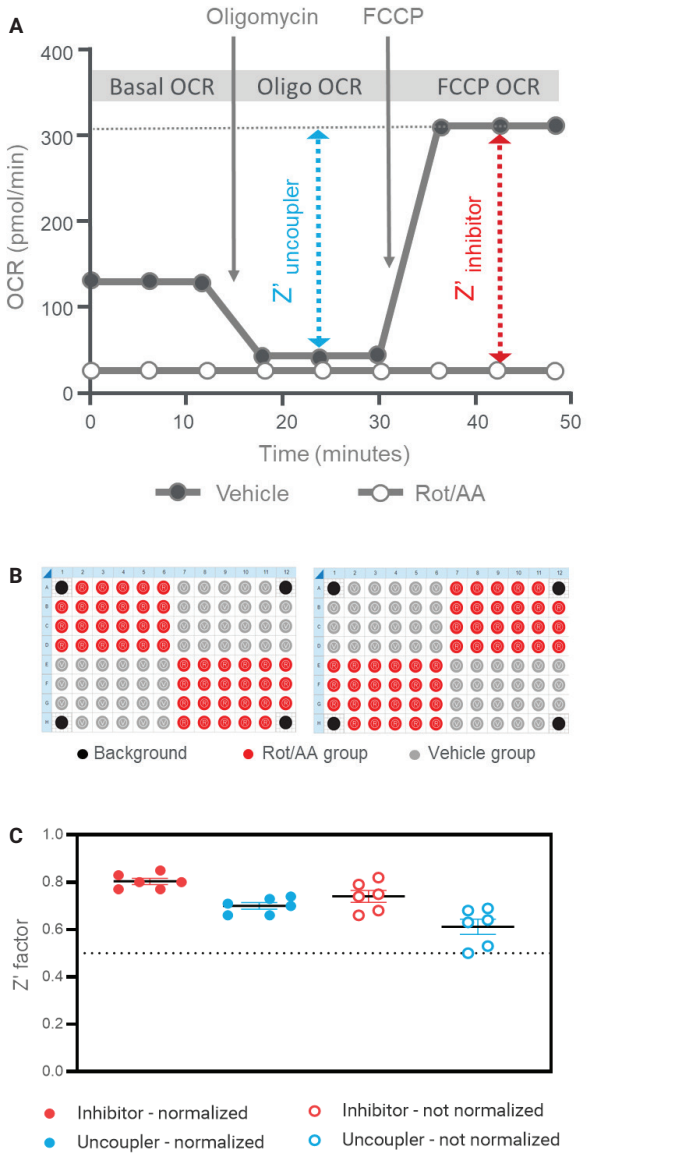


Figure 6. Assessment of Agilent Seahorse XF Mito Tox assay performance using Z'. (A) Dynamic ranges used for calculating Z' for uncoupling (blue) and inhibition (red). (B) Plate map showing the location of control groups (n = 46 per plate); vehicle (gray) and Rot/AA (red). (C) Summary of Z' obtained over three consecutive days with data that was normalized to cell numbers counted by the Agilent Seahorse XF Imaging and Normalization system, or data that was not normalized.

Application of the XF Mito Tox assay for screening of compounds

The XF Mito Tox assay is designed to be used for compound screening, with the ability to screen up to 80 individual compounds per plate at a single concentration (Figure 7A). The assay design may be customized for the desired number of sample compounds and replicate wells (e.g., 10 test compounds with eight replicates per compound, Figure 7B).

To provide initial proof of principle and demonstrate the XF Mito Tox assay, a defined set of compounds known to elicit mitochondrial toxic effects (including inhibition, uncoupling, and OxPhos inhibition) were evaluated (Table 3).

The compounds (100 µM final concentration) were applied to HepG2 cells upon exchange of cell culture medium to XF assay medium and the XF Mito Tox assay was performed. Using Seahorse Analytics, kinetic OCR data was transformed to MTI values and/or analyzed for potential OPI for each compound (Figure 8A to 8D).

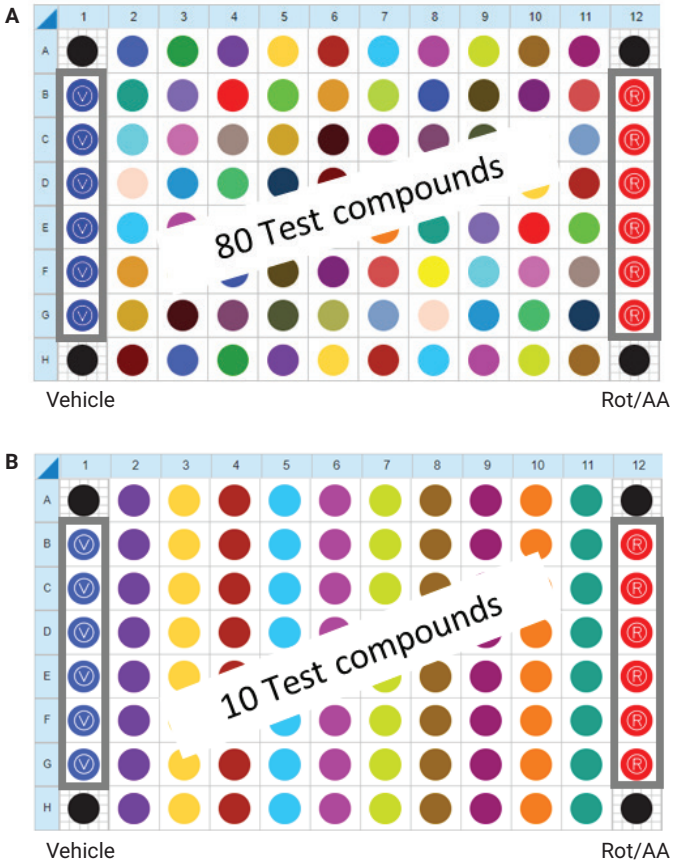
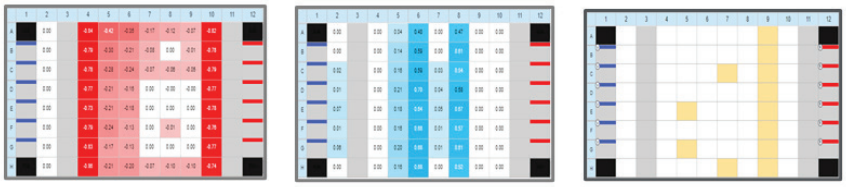


Figure 7. Agilent Seahorse XF Mito Tox assay designs for compound screening. (A) Screening assay design for 80 compounds, n = 1, at a single dose per plate. (B) Screening assay design example for 10 compounds, n = 8, at a single dose per plate. Note that both vehicle and Rot/AA groups are required for calculation of MTI and Z' values.

Table 3. Compound panel used for initial proof-of-concept tests for the Agilent Seahorse XF Mito Tox assay.

Compound (Abbreviation)	Pharmaceutical Intent	Mitochondrial Toxicity	References
Azathioprine (AZ)	Immunosuppressive	Mitochondrial injury, depletion of ATP	Al Maruf *et al.*[13] Menor *et al.*[14]
Clofilium tosylate (CT)	K+ channel blocker.	DNA-stabilizing compound that may be an effective pharmacological alternative for the treatment of POLG-related diseases	Pitayu *et al.*[15]
DCCD (DC)	Inhibitor of ATP synthase.	DCCD inhibits ATP synthase by binding to one of the c subunits and causing steric hindrance of the rotation of the F_o subunit	Luz *et al.*[16] Hong and Pedersen[17]
Diflunisal (DI)	Diflunisal is a salicylic acid derivative with analgesic and anti-inflammatory activity (NSAID)	Uncouples ETC from OxPhos	Nadanaciva *et al.*[18]
Fenofibrate (FF)	Treat severe hypertriglyceridemia and mixed dyslipidemia	Induces mitochondrial dysfunction by decreasing MMP and inducing oxidative stress	Chen *et al.*[19]
Nimesulide (NI)	Anti-inflammatory drug, NSAID	Uncouples mitochondria and induces mitochondrial permeability transition in human hepatoma cells: protection by albumin	Nadanaciva *et al.*[18]
Pentamidine isethionate (PI)	Pentamidine is an antimicrobial medication	Mitochondrial inhibitor	Ludewig, *et al.*[20]
Risperidone (RI)	Antipsychotic: it can treat schizophrenia, bipolar disorder, and irritability caused by autism.	ETC inhibition (CI and CII/III)	Cikánková *et al.*[21]

A



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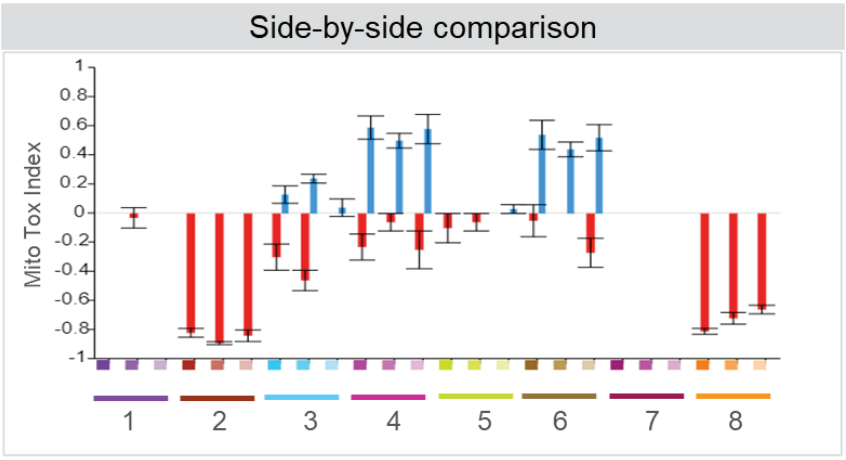

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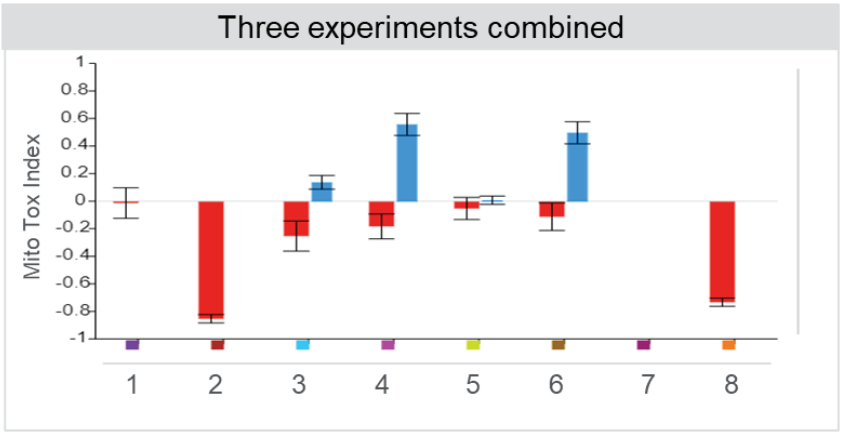


D

	Compound	Inhibitor	Uncoupler	OPI
1	Azathioprine			
2	Clofilium tosylate	–0.85 ± 0.03		
3	DCCD	–0.31 ± 0.11	0.14 ± 0.05	
4	Diflunisal	–0.18 ± 0.09	0.56 ± 0.08	
5	Fenofibrate			
6	Nimesulide	–0.11 ± 0.10	0.50 ± 0.08	
7	Pentamidine isethionate			
8	Risperidone	–0.73 ± 0.03		

Mean ± SEM

Figure 8. Agilent Seahorse XF Mito Tox assay: initial proof of principle. (A) MTI heat map views displaying inhibitor (left) and uncoupler (middle) MTI values, and detection of OPIs (right). (B) MTI bar chart showing side-by-side comparison of MTI values from three independent experiments for each compound. (C) Bar chart showing mean MTI values from three independent experiments. (D) Summary table of MTI values and OPI detection for each test compound (mean + SEM); (n = 8).

Data analysis of the XF Mito Tox assay provided the following results: clofilium tosylate and risperidone showed significant negative MTI values, indicating inhibition as the mechanism of mitochondrial toxicity, and in agreement with Pitayu *et al.*[15] and Cikánková *et al.*[21], respectively. Diflusinal and nimesulide resulted in significant, positive MTI values, indicating uncoupling as the mechanism of mitochondrial toxicity, in agreement with Nadanaciva *et al.*[18] DCCD, a known, less-specific ATP synthase inhibitor (Ludewig, *et al.*[20]) has uncoupling and inhibitory effects, which may be due to nonspecific effects exhibited at the relative higher concentration used here (100 µM). Two compounds, azothioprine and fenofibrate, showed no significant changes in MTI value. In the case of azathioprine at 100 µM, the toxic effects were minimal, however, detection of toxic effects would be expected with increasing the concentration of azathioprine, as reported by Al Maruf *et al.*[13] In the case of fenofibrate, Chen *et al.*[19] demonstrated a significant decrease in the OCR when provided to cells; however, the time of compound treatment was 24 hours versus 2 hours as described in this study, and thus detection of toxic effects would be expected with increasing the time of incubation with the compound before performing the XF Mito Tox assay. Effects of dose and time of compound treatment are discussed further in this application note, and are outlined in the Agilent white paper by Rogers *et al.*[8]

As noted above, a specific case of mitochondrial toxicity due to decreased mitochondrial function is the direct inhibition of a component of the OxPhos machinery: ATP synthase, ANT, and/or P_iT, which often shows a significant decrease in Basal OCR, while Oligo OCR and FCCP OCR are not significantly affected (Figure 5). This result is exemplified when pentamidine isethionate is used as the test compound (Figure 9). In these types of cases, the XF Mito Tox assay data analysis will detect and report potential OxPhos inhibition using the OPI detection heat map. Further details of OPI detection criteria may be found in the Agilent white paper by Rogers *et al.*[8]

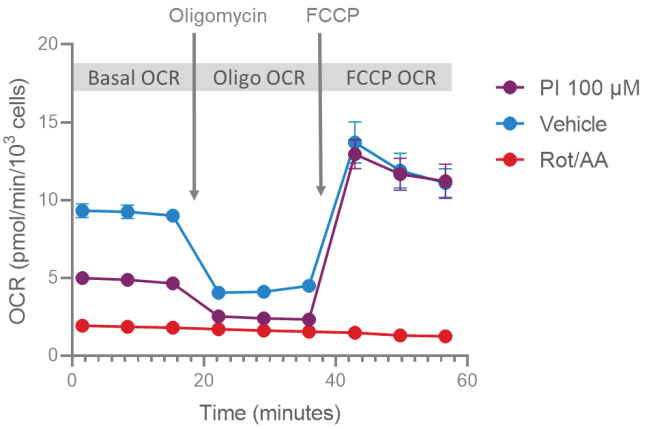


Figure 9. Example of OxPhos inhibition: Kinetic trace of the Agilent Seahorse XF Mito Tox assay showing control groups (vehicle and Rot/AA) and pentamidine isethionate treatment. Note that a significant decrease in Basal OCR but not FCCP OCR is observed.

The small-scale example tests described above with toxic compounds known to affect mitochondria allowed for primary establishment and initial testing of the assay design and MTI parameter, and provided the expected results under conditions used. To provide further confidence in the MTI value as a robust parameter for detecting mitochondrial toxicity, the XF Mito Tox assay was compared to the "Glu/Gal" method, a common and accepted technique for assessing mitochondrial toxicity in cultured cell models.[5,12,22] The Glu/Gal method relies on providing either glucose or galactose in the culture and/or assay media, with galactose making the cells more reliant on mitochondrial ATP, and thus more susceptible to mitochondrial toxicity. In brief, increasing concentrations of test compounds (i.e. dose-response assays) are provided to cells in either glucose or galactose media, then total cellular ATP content is measured. Dose-response curves are then generated to obtain IC_{50} values, and relative differences in IC_{50} values between Glu and Gal conditions are subsequently used to assess mitochondrial toxicity (described in detail in the Experimental section and Marroquin *et al.*[12], Hynes *et al.*[5]). In summary, the Glu/Gal method uses a cell viability assay based on total cellular ATP measurements under different culture conditions to indirectly assess mitochondrial toxicity.

To this end, a panel of 17 well characterized mitochondrial toxicants (including those from the above experiments) were tested using both the XF Mito Tox and the Glu/Gal assays. The results are summarized in Figure 10.

A

Compound	XF Mito Tox Assay			Glu/Gal Assay
	Inhibitor	Uncoupler	OxPhos Inhibitor	
Antimycin A				
Amiodarone				
Azathioprine				
Clofilium Tosylate				
*Chlorpromazine				
*Diflunisal				
Fenofibrate				
Flutamide				
Ketonocazole				
Nabumetone				
*Nimesulide				
Nimodipine				
Oligomyicin				
Pentemidine				
Risperidone				
Rotenone				
*Tamoxifen				

B

Toxicity	MTI		OPI	Glu/Gal
Yes	≤ –0.5	>= 0.5	Yes	≥ 3
Maybe	≤ –0.2	>= 0.2	No	≥ 2
Not significant	> –0.2	< 0.2		< 2

Figure 10. Summary and comparison of Agilent Seahorse XF Mito Tox assay and Glu/Gal total ATP assay. (A) Results of compound screening for mitochondrial toxicity using XF assay versus Glu/Gal method. (B) Mitochondrial toxicity "hit" criteria for XF Mito Tox assay results (MTI and OPI) and Glu/Gal assay results. *Indicates compounds identified "yes" only by the XF Mito Tox assay.

The results indicate that when compared to Glu/Gal assay (ratio ≥3), the XF Mito Tox assay identifies more instances of significant mitochondrial toxicity (9/17 versus 4 /17), as well as having the distinct advantage of providing preliminary information as to the type of toxicity (inhibition, uncoupling, and/or OPI). While both assays readily detected known potent mitochondrial toxicants (e.g. antimycin A, oligomycin, pentamidine isethionate, risperidone), only the XF assay detected nine other compounds with significant mitochondrial toxicity (MTI ≤–0.5 or MTI ≥0.5), showing both inhibition and uncoupling.

In summary, when compared with the Glu/Gal method, the XF Mito Tox assay identifies more instances of mitochondrial toxicity, or conversely, Glu/Gal underestimates instances of mitochondrial toxicity. Further, the XF Mito Tox assay can suggest a mode of toxicity (inhibition, uncoupling, or OPI), while the Glu/Gal method cannot. It should be noted that this workflow does not require two types of culture/medium conditions (Glu versus Gal) and can be performed at single dose for initial screening, while the Glu/Gal method requires both media types and generation of IC_{50} value for comparison. Finally, the XF Mito Tox assay measures mitochondrial function directly (OCR), while the Glu/Gal does use an indirect analyte, total cellular ATP, which can be more indicative of overall cytotoxicity, rather than specific mitochondrial toxicity. In summary, the XF Mito Tox assay provides a more direct and specific assessment of mitochondrial toxicity when compared to the Glu/Gal method.

To further demonstrate the ability of the XF Mito Tox assay to rapidly screen test compounds at mid to high throughput, the workflow was applied to a library of 240 hepatotoxic compounds (SCREEN-WELL Hepatotoxicity library, Enzo Life Sciences). The compound library was divided into three plates of 80 compounds per plate, and the XF Mito Tox assay was performed for the entire library at a single dose on the same day (three assay plates/day). This strategy was repeated across three days, providing triplicate values for each compound. The Bravo platform was used to automate several steps of the assay (see the "Experimental" section). Kinetic OCR data were transformed into MTI values and results were further collated based on arbitrary MTI value thresholds defining no/low, medium, and high probability of mitochondrial toxicity (Figure 11).

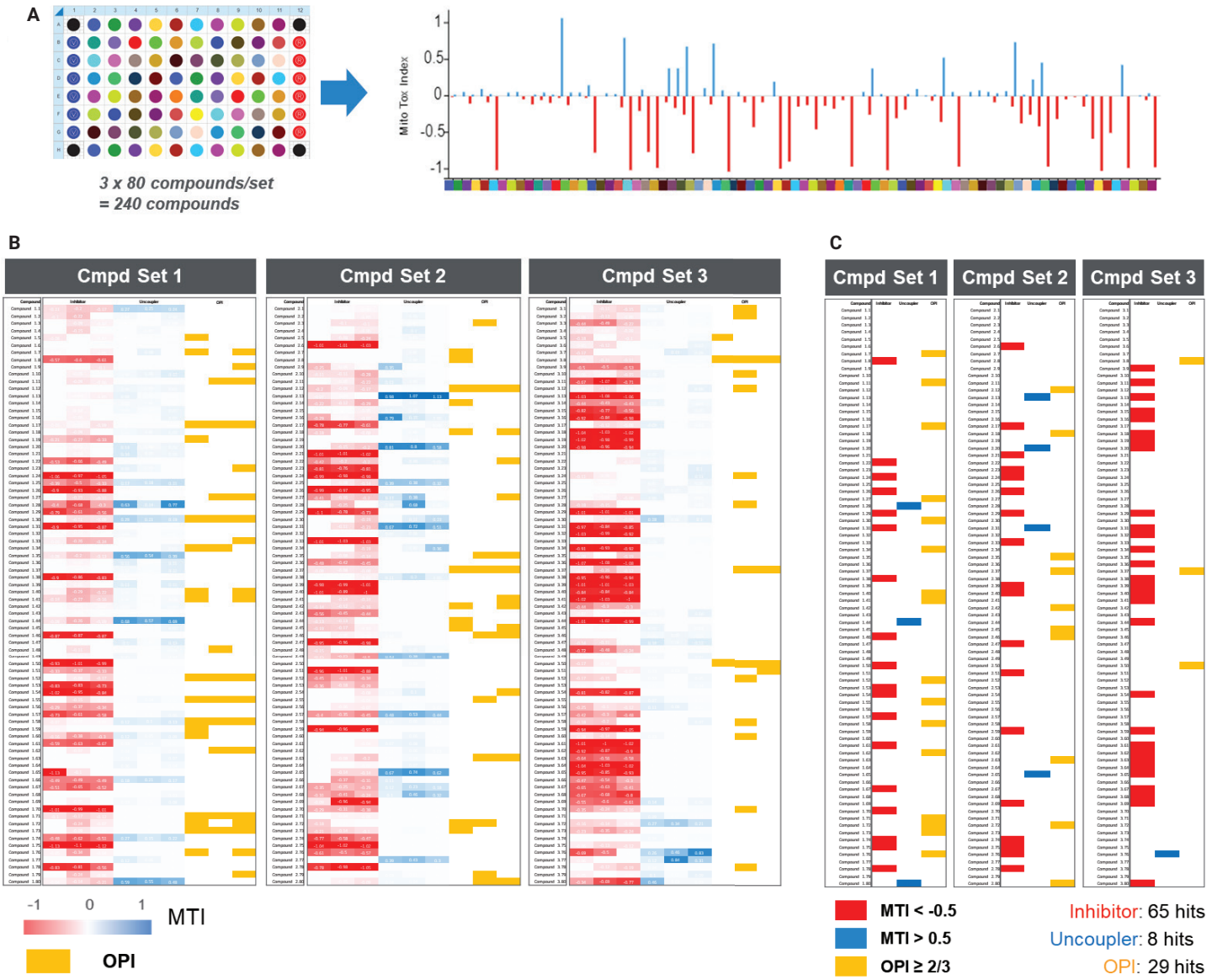


Figure 11. Results of 240 compound library screen. (A) Representative Agilent Seahorse XF Mito Tox assay plate map design (left) for 80 compounds and requisite control groups (vehicle and Rot/AA) and resulting MTI bar chart (right). (B) MTI values for the entire data set (80 compounds per plate, for total of 240 compounds per day, repeated across 3 days for total of 9 plates, n = 3 per compound), showing side-by-side comparison of MTI values and/or OPI detection among replicating plates for the 3 compound (cmpd) sets. (C) Average of triplicate MTI values for the entire data set. MTI reporting thresholds ≥0.5 and ≤−0.5 for uncoupling and inhibition, respectively. OPI reporting threshold = 2/3 (i.e., reported as OPI if a compound is detected as OPI in two out of the three replicating experiments).

Dose-response evaluation of mitochondrial toxicity

Screening compounds using the XF Mito Tox assay provides a rapid way to assess the type and magnitude of mitochondrial toxicity for a larger number of samples. Once the initial assessment of toxicity is established for a single dose, and depending on the goals of the investigation, compounds of further interest may then be evaluated through dose-response assays. This provides a secondary layer of information that may not be evident from single-dose analysis. As noted above, MTI values are calculated for each test sample/well, allowing this value to also be used for generating dose-response graphs, including dose-response bar charts, curves, and IC_{50}/EC_{50} values. Figure 12 illustrates an XF Mito Tox assay performed as dose-response. Details of curve fitting and IC_{50}/EC_{50} determination are described in the Agilent white paper by Rogers *et al.*[8]

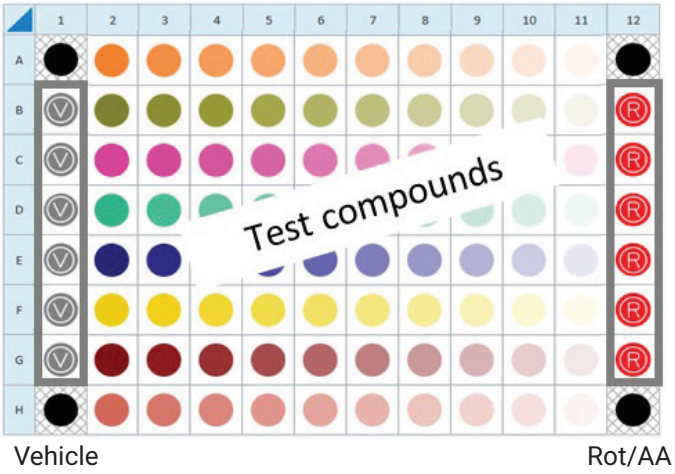


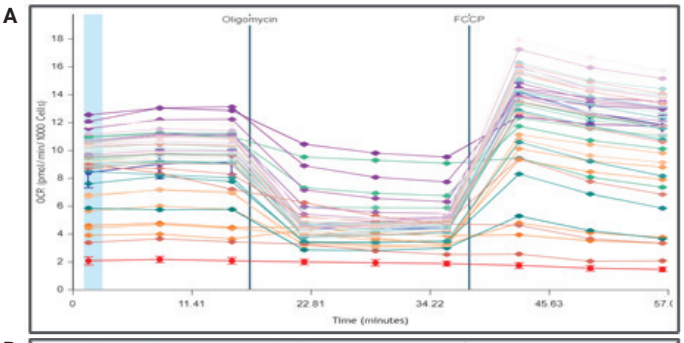
Figure 12. Agilent Seahorse XF Mito Tox assay design for dose-response analysis. Eight compounds, ten doses per compound, n = 1, per plate. Note that both vehicle and Rot/AA groups are required for calculation of IC_{50} and/or EC_{50} values.

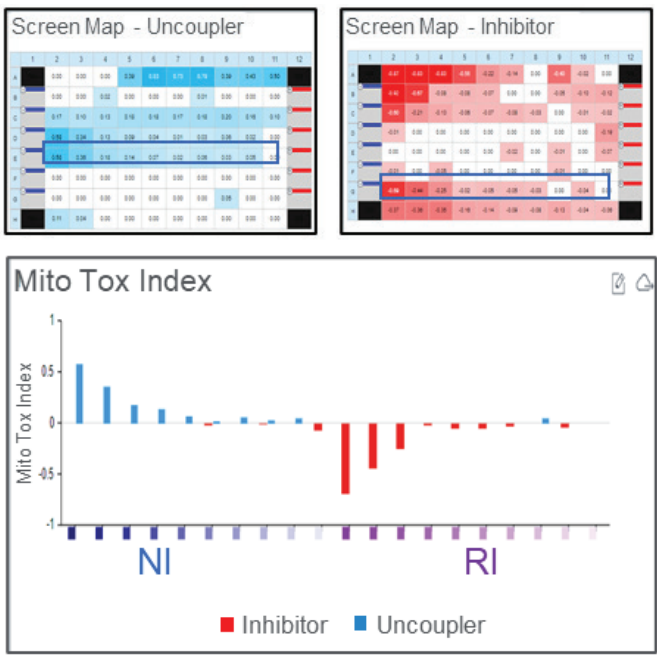
As a demonstration of the application of MTI values in data analysis for a dose-response assay, experiments with five known compounds were performed. Figure 13A shows kinetic dose-response OCR data for the five compounds, which were then transformed to MTI values for each dose using Seahorse Analytics. The MTI values are then plotted against compound concentration, yielding dose-response curves with calculated IC_{50} (or EC_{50}) values (Figures 13B and 13C). Note that in some cases, the mode of mitochondrial toxicity can be dose-dependent, and is discussed in detail with examples in the Agilent white paper by Rogers *et al.*[8]

Workflows and considerations for XF Mito Tox assay optimization

A summary of the XF Mito Tox assay as a complete solution for the quantitative assessment of mitochondrial toxicity, from design to data interpretation, is illustrated in Figure 14.

The schematic diagram in Figure 2 illustrates three possible workflows for performing the XF Mito Tox assay. All are identical except for how and when the test compound is provided to the cells. The first method uses the injection ports to deliver the compound *in situ* (Figure 2A). This method





F

Compound	IC_{50}	EC_{50}
Clofilium Tosylate	3.507e-5	NA
Diflunisal	N/A	5.432e-5
Nimesulide	N/A	4.235e-5
Risperidone	4.117e-5	NA
Tamoxifen	3.541e-5	N/A

Figure 13. Dose-response evaluation of five mitochondrial toxic compounds using MTI values. (A) Kinetic OCR measurements. (B) Dose-response curves using MTI values for inhibitors, with IC_{50} values. (C) Dose-response curves using MTI values for uncouplers, with EC_{50} values. (D) and (E) MTI heat maps and bar chart, respectively. These widgets may also be applied to XF Mito dose-response assay designs, providing alternative options to illustrate results from XF Mito Tox assay dose-response experiments. (F) Mean IC_{50} and EC_{50} values calculated from triplicate data (three plates).

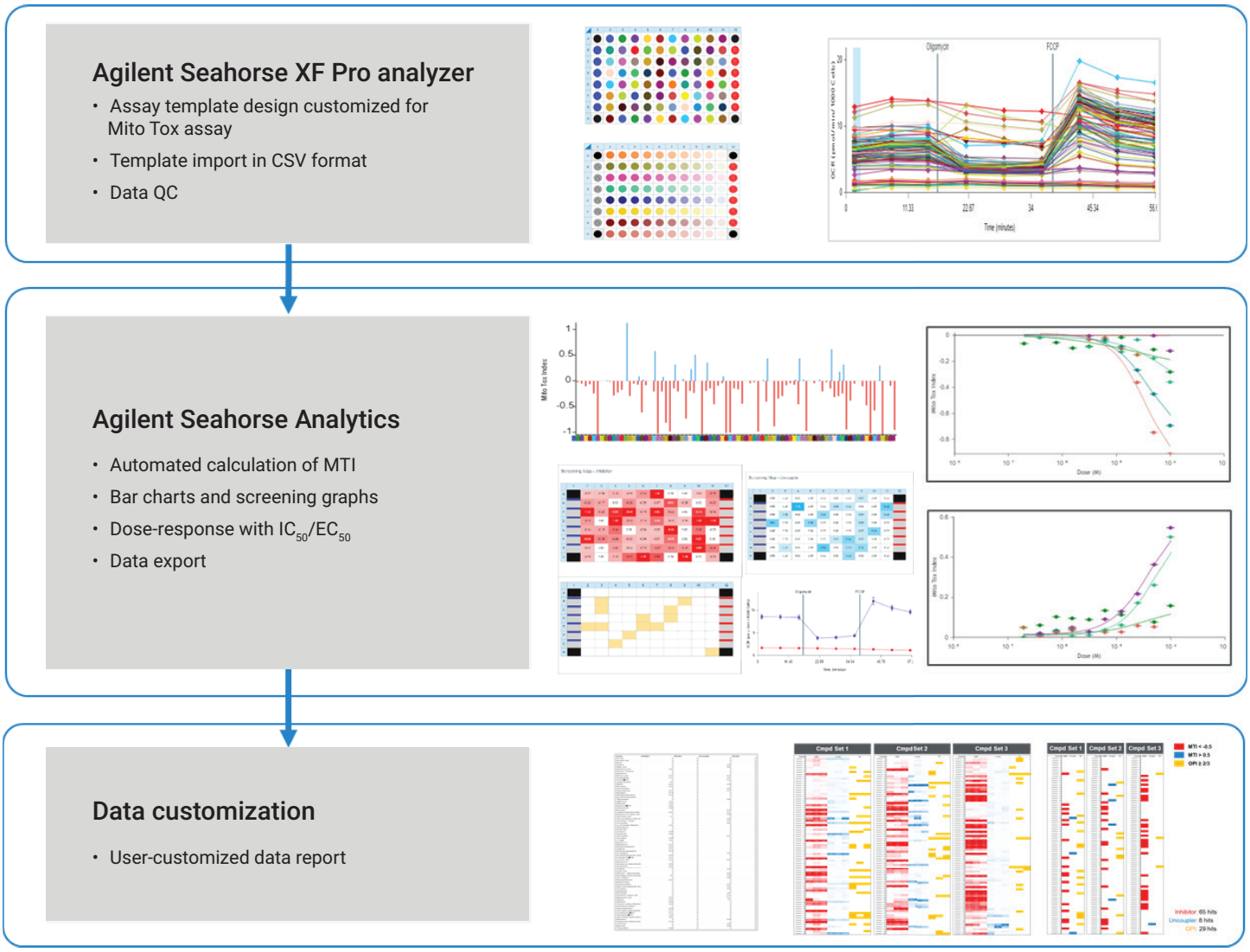


Figure 14. Agilent Seahorse XF Mito Tox assay workflow summary, including assay design and resulting data interpretation through Agilent Seahorse Analytics and further data customization.

offers the advantage of kinetically tracking any acute effects of the compound in real time, while also providing XF Mito Tox assay parameters. The second, and often simplest method, provides test compounds to the cells when the cell culture medium is exchanged for XF assay medium (Figure 2B), allowing short-term compound effects to be assessed (1 to 2 hours). It is recommended to start with this workflow when investigating mitochondrial toxicity. The workflow may also be adapted for extended compound exposure (>4 hours), as outlined in Figure 2C. While this offers the ability to perform long-term compound treatments, it does require the compound to be added again when the growth medium is exchanged for assay medium.

Table 4 provides some guidance and consideration for the application of each workflow.

The primary variables to consider for this assay are the concentration of the compound(s) and the length of time the cells will be exposed to the compound(s). The degree of compound solubility and/or the ability to permeate the plasma/mitochondrial membranes must also be considered (if possible). For compounds that exhibit greater solubility/cell permeability, these may be used at relative higher concentrations for short periods of time when performing an initial assessment (acute or short method). For less soluble/cell permeable compounds, lower concentrations may require longer incubation periods to elicit significant toxic effects. Note that the apparent mechanism of toxicity may be influenced by the length of time of compound exposure as discussed in further detail in the Agilent white paper by Rogers *et al.*[8]

In addition to the elements described previously for assay design, the following factors should be considered before and/or during performance of XF Mito Tox assays.

- **Cell density:** Optimization of cell density must be performed.
- **Concentration of FCCP:** Optimization of FCCP must be performed to ensure the greatest separation of positive and negative controls (maximize the dynamic range of the assay).
- **Assessment of assay performance (Z'):** Once cell density and FCCP concentration are optimized, and before starting screening or dose-response assays, it is suggested that assessment of assay be performed using the Z' metric to demonstrate consistent assay performance from plate to plate/day to day, as is exemplified in Figure 10. The Z' values can then be used to track and assess the quality of assay performance for screening and dose-response assays.
- **Normalization:** While normalization of the resulting data to cell number in each well is not required, it is encouraged for achieving more consistent intraplate measurements, especially for experiments with longer culture/growth and/or compound exposure times. Note, however, that normalization to cell number may alter data interpretation and/or mask cytotoxic effects of compounds being tested. For more information, please refer to the Agilent white paper by Rogers, *et al.*[8]
- **Cell viability and/or cytotoxicity:** Changes in cell viability and/or cytotoxic effects induced by compounds can affect XF Mito Tox assay results and downstream interpretation.[8] Orthogonal assessment of cell viability and/or cytotoxic effects may be required to ensure accurate assessment and interpretation of XF Mito Tox assay data.

Table 4. Comparison and considerations for choosing the proper Agilent Seahorse XF Mito Tox assay workflow.

	Method of Compound Provision to Cells		
	Acute Injection	**Short-Term Incubation (1 to 2 Hours)**	**Long-Term Incubation (>4 Hours)**
MTI Calculated?	Y	Y	Y
IC_{50}/EC_{50} Reported?	Y	Y	Y
Real-Time Drug Kinetics?	Y	N	N
Compound Pretreatment Required?	N	Y	Y
Second Compound Treatment Required?	N	N	Y
Best For	Faster-acting drugs at lower relative concentrations, assessing drug kinetics (slow versus fast acting).	Slower-acting drugs at higher relative concentrations.	Slower acting drugs at lower relative concentrations.
Considerations	An 8x final drug concentration is required for the injection port.	Often, simpler to perform for initial assessment and/or dose-response assays.	Data quality may be affected due to extended cell culturing and/or the more prevalent cytotoxicity becomes, effects of drug metabolism, and indirect mitochondrial toxicity.

Conclusion

This application note presents a customized XF assay, the Agilent Seahorse XF Mito Tox assay, to assess mitochondrial toxicity. This assay demonstrates the ability to identify and specifically distinguish among different modes of mitochondrial toxicity with high sensitivity. This assay introduces and provides a standardized quantitative measurement of the magnitude of the toxicity, the MTI, useful for both screening and dose-response types of assays. In addition, to provide assurance of resulting data quality, a metric of assay performance is provided (Z'). This assay design and the respective output parameters enable rapid, straightforward implementation and intuitive, confident data interpretation when examining mitochondrial toxicity of therapeutic compounds, assisting identification of mitochondrial liability and reducing risk in drug pipelines.

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Printed in the USA, April 27, 2022
5994-4778EN

RA44666.5719212963

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