Application Note

Cell Analysis

Agilent

Trusted Answers

Measurement of Mitochondrial Toxicity Using Primary Hepatocytes

The Seahorse XF Mito Tox assay workflow to assess drug-induced mitochondrial dysfunction

Authors

Y. Kam, L. Winer, G. W. Rogers, and N. Romero
Agilent Technologies, Inc.

Abstract

Mitochondrial dysfunction is known to play a central role in drug-induced hepatotoxicity, and thus early detection of mitochondrial toxicity is a critical component of initial drug development efforts. While immortalized cell lines are widely used for *in vitro* assessment of hepatotoxicity, cryopreserved primary human hepatocytes are a preferred model for drug safety screening. This application note describes the use of human primary hepatocytes in the context of the Agilent Seahorse XF Mito Tox assay, including assay optimization, validation, and representative data.

Introduction

The Agilent Seahorse XF Mito Tox assay kit enables streamlined, sensitive detection and characterization of mitochondrial toxicants in conjunction with the Agilent Seahorse XF Pro analyzer and dedicated data analysis tools. This assay allows discrimination between different modes of mitochondrial toxicity and delivers a standardized quantitative parameter, the Mito Tox Index (MTI), indicating the magnitude of the toxicity. It also provides a metric, Z', to evaluate assay quality and performance.[1,2]

Immortalized hepatic cell lines (e.g., HepG2 and HepaRG) are widely applied for *in vitro* assessment of drug-induced liver injury (DILI) due to convenience in cell propagation, cost, and data consistency. However, researchers often prefer to use primary cultured hepatocytes or induced pluripotent stem cell-originated hepatocytes as a more relevant predictive model of DILI.

This application note describes the recommended workflow for assessing mitochondrial toxicity in primary human hepatocytes including optimization steps required for the Seahorse XF Mito Tox assay (Figure 1). This workflow includes optimization of two critical experimental variables, cell seeding density, and FCCP concentration. Both variables are required to obtain optimal assay performance. Results of assay optimization, and examples of detection of mitochondrial toxic compounds using primary hepatocytes are presented. Although this workflow is demonstrated using primary human hepatocytes, it can be applied to any type of primary cell or immortalized cell line for use with the Seahorse XF Mito Tox assay.

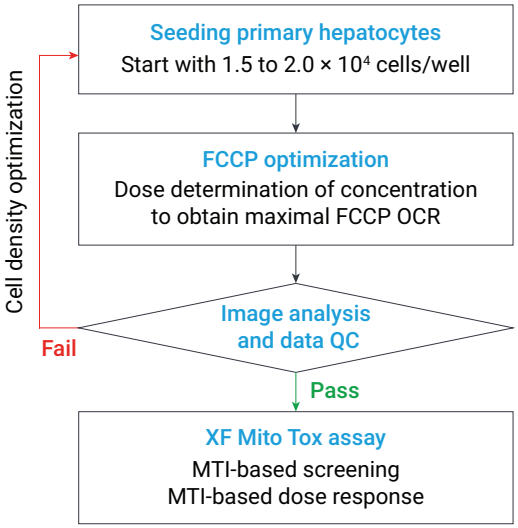

Figure 1. Optimization workflow for the Agilent Seahorse XF Mito Tox assay using primary human hepatocytes, including cell density optimization, FCCP concentration optimization, image analysis, and data quality control.

Experimental

Materials

Table 1. Key products required for the Agilent Seahorse XF Mito Tox assay.

Item	Part No.
Agilent Seahorse XF Pro Analyzer with XF Discovery License	S7855A
Agilent Seahorse XF Mito Tox Assay Kit	103595-100
Agilent Seahorse XF Pro M FluxPak, 18 Assays(1)	103775-100
Agilent Seahorse XF Pro M FluxPak Mini, 6 Assays(1)	103777-100
Agilent Seahorse XF Pro M Cell Culture Microplate(2)	103774-100
Agilent Seahorse XF DMEM Medium pH 7.4	103575-100
Agilent Seahorse XF 1.0 M Glucose Solution	103577-100
Agilent Seahorse XF 100 mM Pyruvate Solution	103578-100
Agilent Seahorse XF 200 mM Glutamine Solution	103579-100

(1) Part numbers 103775-100 and 103777-100 contain Agilent Seahorse XF sensor cartridges and Seahorse XF Pro M cell culture plates.
(2) Part number 103774-100 does not contain XF sensor cartridges, which are also required to perform XF assays. This part number is only needed when extra plates are required for optimizing seeding density and/or FCCP concentration.

Cryopreserved human hepatocytes, plateable (HUCPG), human cryopreserved hepatocyte thawing medium (MCHT50), and hepatocyte plating medium (MP100) were purchased from Lonza. Test compounds azathioprine, clofilium tosylate, nimesulide, and pentamidine isethionate were purchased from Sigma-Aldrich.

Cell culture

Primary hepatocytes were cultured according to manufacturer's methods. Agilent Seahorse XF Pro M cell culture plates were coated by adding 20 µL of a 50 µg/mL solution of collagen (Enzo, ALX-522-435) to plate wells. The cell plates were incubated at 37 °C in a non-CO_2 incubator for two hours. Wells were then washed twice with 200 µL sterile H_2O and allowed to air-dry for at least 30 minutes before cell seeding. Unused coated plates were stored at 4 °C for up to 1 week.

Primary hepatocytes were thawed in the thawing medium, centrifuged, and the pellet resuspended in plating medium. Cells were counted using a Vi-CELL Analyzer (Beckman Coulter) and seeded in Seahorse XF Pro M cell culture plates coated with collagen at a density of 2 × 10^4 cells per well (or as indicated in each figure). Cells were then incubated at 5% CO_2 at 37 °C in a humidified atmosphere for 24 hours. In accordance with manufacturer's recommendations, cell plates were not left at room temperature after seeding to maximize cell viability. To prevent loss of viability, cells should not be centrifuged onto the cell plate.

On the following day, cells were washed twice with Agilent Seahorse XF DMEM medium, pH 7.4 supplemented with 10 mM Seahorse XF glucose solution, 1 mM Seahorse XF pyruvate solution, and Seahorse XF 2 mM glutamine solution (final concentrations). Compound solutions of azathioprine, clofilium tosylate, nimesulide, pentamidine isethionate (final concentrations of 0.2 to 100 µM), and rotenone/antimycin A (R/AA) (final concentrations of 0.5 µM each) were added after cell washing. The plate was then incubated at 37 °C, without CO_2, for 60 minutes. Cell plates were then transferred to the XF Pro analyzer for XF Mito Tox assay performance.

Seahorse XF Mito Tox assay

All Seahorse XF Mito Tox assays were performed as described in the Agilent Seahorse XF Mito Tox assay kit user guide, including preparation of compounds, reagents, and sensor cartridges.

Normalization

After performing the XF assay, cell plates were imaged using the Agilent Seahorse XF Imaging and Cell Counting software with an Agilent BioTek Cytation 1 cell imaging multimode reader to determine the cell numbers in each well, which were used to normalize assay measurements.

Assay design, data QC, and data analysis

Agilent Seahorse Wave Pro Controller software was used to generate all assay templates, including dose-response assays, and to perform data quality analysis. Agilent Seahorse Analytics XF Mito Tox data analysis widgets were used to report and visualize Z' values, MTI values, dose-response curves, and IC_{50} and EC_{50} values.

Results and discussion

Optimization of cell seeding density and FCCP concentration

The optimal seeding density for human primary hepatocytes was initially estimated by considering the surface area of an XF Pro M plate well. Per manufacturer's instructions (Lonza), it is recommended to use 5×10^4 cells per well, using a conventional 96-well microplate. Because the surface area of each well in an XF Pro M plate is approximately 40% of the surface area of a conventional 96-well microplate, an initial cell seeding density of 2×10^4 cells per well was calculated.

The XF Mito Tox assay was performed using primary hepatocytes seeded at five different densities (1, 1.5, 2, 3, and 4×10^4 cells per well) to provide seeding density values both above and below the estimated optimal seeding density of 2×10^4 cells per well. In this study, 1 µM FCCP was used as an initial estimated concentration, and will be further optimized as described below.

Figure 2A illustrates Mito Tox assay kinetic OCR output and shows typical responses to oligomycin and FCCP injections. Figure 2B shows OCR versus cell density and indicates increasing OCR with increasing cell density for basal and FCCP OCR, up to 3×10^4 cells per well, with decreases in basal and FCCP OCR when using 4×10^4 cells per well. The brightfield images obtained by Seahorse XF Imaging and Cell Counting software showed that cells were nearly confluent at a seeding density of 2×10^4 cells per well, with a consistent monolayer and a minimal number of cell clusters. In contrast, cells seeded at 4×10^4 cells per well showed overconfluent seeding conditions, and significant instances of cell clusters (Figure 2C).

Taken together, a seeding density of 2×10^4 cells per well is recommended for use of primary human hepatocytes in XF Mito Tox assays because: i) both basal and FCCP OCR are within the linear range of response with respect to an increase in seeding cell density, ii) the FCCP OCR for 2×10^4 cells per well (~350 pmol/min) is within the validated OCR performance range for XF Pro instruments, and thus, iii) it provides the largest signal window (Δ >300 pmol/min between FCCP and R/A OCR) for assessing mitochondrial toxicity. In addition, using the largest signal window increases the probability of obtaining acceptable (i.e., high) Z' values.

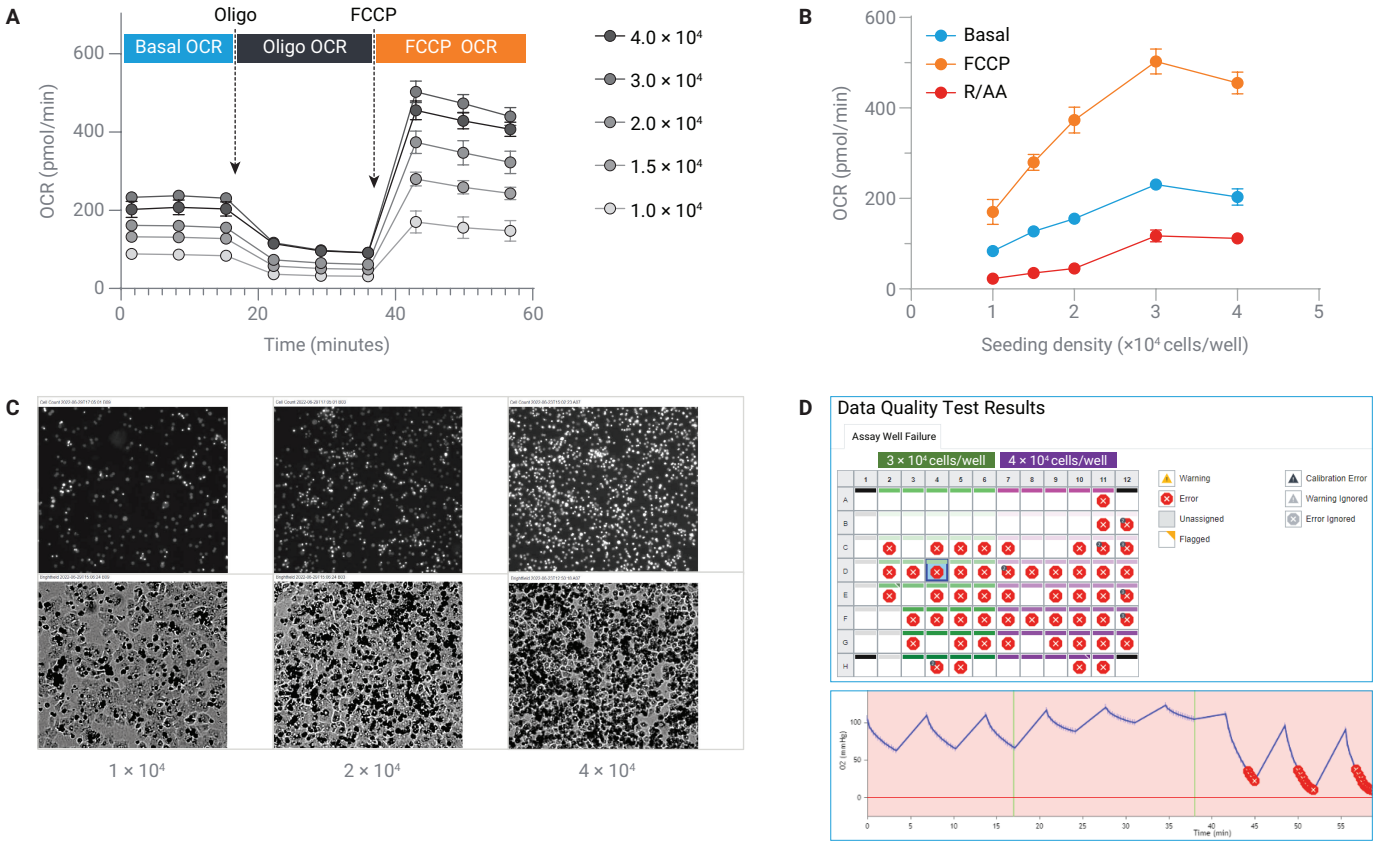

Figure 2. Optimization of primary hepatocyte density for the Agilent Seahorse XF Mito Tox assay. (A) Agilent Seahorse XF Mito Tox assay kinetic OCR data with primary hepatocytes seeded as indicated (mean ±S.D., n = 6 to 12, R/AA groups omitted for clarity). (B) OCR versus cell density for Basal OCR (panel A, rate 2), FCCP OCR (panel A, rate 7) and R/AA OCR. (C) Fluorescence (top) and bright field (bottom) images of a representative well for 1, 2, and 4 × 10^4 cells per well. (D) Agilent Seahorse XF Pro software data quality output showing error warnings and representative low O_2 level data (O_2 level <40 mmHg) for 3 × 10^4 cells/well (well D4).

Optimal seeding density selection is further supported by data quality analysis of the assay data for potential technical issues (Figure 2D). For 3 and 4 × 10^4 cells per well, the majority of wells showed errors indicating that O_2 levels dropped below the recommended minimum level of 40 mmHg during some XF measurements. In contrast, no low-O_2 level errors were observed for 1 to 2 × 10^4 cells per well (data not shown).

Next, the concentration of FCCP, used to elicit maximal respiration in the XF Mito Tox assay, was optimized. This step is critical, as suboptimal FCCP concentrations will result in a decreased signal window for detection of toxicity, as well as decreased Z' values. Figure 3 shows the results of an FCCP titration using primary hepatocytes at the optimized seeding density of 2 × 10^4 cell per well. The results show similar FCCP OCR values (~40 pmol/min/1,000 cells) over a broad range (0.5 to 2.5 µM) of FCCP, which was centered at ~1.5 µM. This was therefore chosen as the optimal FCCP concentration.

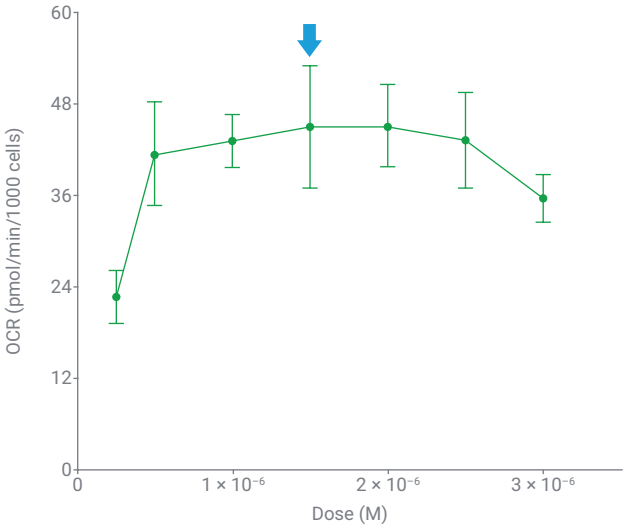

Figure 3. Optimization of FCCP concentration. Normalized FCCP OCR versus FCCP concentration. Maximal FCCP OCR is achieved between 0.5 and 2.5 µM FCCP, centered at 1.5 µM FCCP (grey arrow). Mean ±S.D., n = 6.

Since an FCCP concentration of 1 µM used in the cell seeding density optimization (Figure 2) shows nearly identical FCCP OCR values (~40 pmol/min/1,000 cells), a cell density of 2×10^4 cells per well remains optimal.

Finally, to ensure that optimal selected conditions (2×10^4 cells per well and an FCCP concentration of 1.5 µM) are consistent across different cell preparations, Seahorse XF Mito Tox assays were performed using different batches (lots) of primary hepatocytes. Results confirmed that the optimized conditions can be widely applied to different lots of primary hepatocytes (data not shown). However, to ensure the most robust assay performance, it is highly recommended to perform optimization of cell density whenever a new lot or batch of primary hepatocytes cells is used.

Evaluation of assay performance using Z'

The Z-prime (or Z') value is a statistical measure widely used to determine the quality of a high-throughput screening assay. It considers the measurable difference between positive and negative controls (i.e., signal window) and the precision (i.e., the standard error) of the controls. Z' values range from 0 to 1, with values >0.5 indicative of a robust screening assay.[5] To assess Seahorse XF Mito Tox assay performance using primary hepatocytes, a Z' evaluation assay was performed as described[1], and Z' values for detection of inhibition and uncoupling were calculated by Seahorse Analytics (Figure 4). Z' values of 0.69 and 0.66 were obtained for detection of inhibition and uncoupling, respectively, indicating that conditions optimized for this lot of primary hepatocytes are well-suited for use in a full-scale, high-throughput Seahorse XF Mito Tox screening assay.

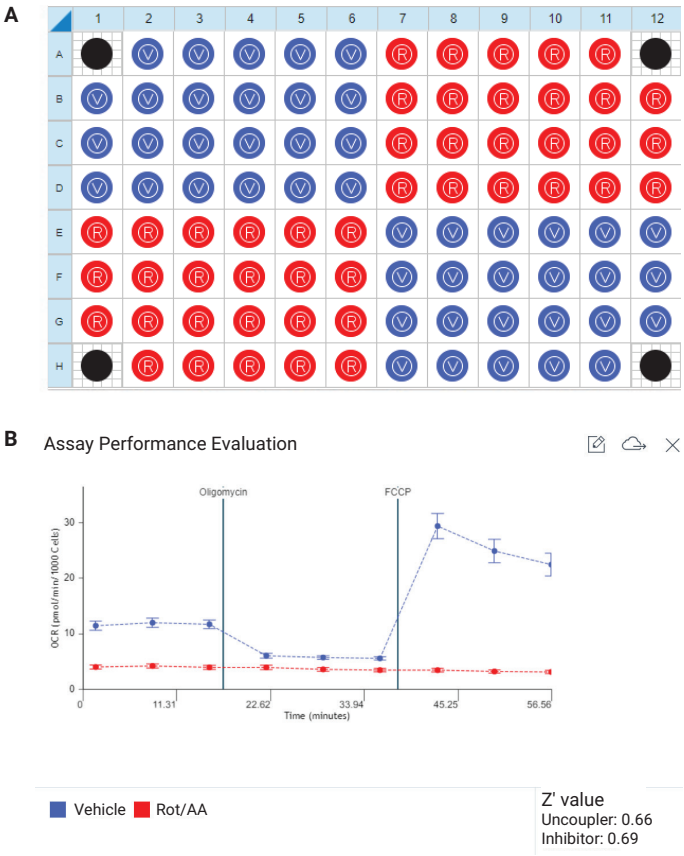

Figure 4. Z' evaluation of primary human hepatocytes in the Agilent Seahorse XF Mito Tox assay. (A) XF Mito Tox assay design for assay performance (Z') evaluation. (B) Data from the assay performance evaluation, using optimal cell density (2×10^4) and FCCP concentrations (1.5 µM). Z' values were calculated via Seahorse Analytics using normalized OCR data. Mean ±S.D., n = 46.

Mitochondrial toxicity screening and dose-response evaluation

To test the Seahorse XF Mito Tox assay with primary hepatocytes, four compounds known to elicit different types of mitochondrial toxicity were selected, and the assay was performed using the optimized conditions determined. These compounds were previously tested for mitochondrial toxicity using HepG2 cells and identified clofilium tosylate as an inhibitor, nimesulide as an uncoupler, and pentamidine isethionate as a potential OxPhos inhibitor. Azathioprine showed no mitochondrial toxic effects at the concentration used.[2]

The results of the Seahorse XF Mito Tox assay performed with primary hepatocytes are illustrated in Figure 5. Figure 5A shows the resulting MTI values for each compound when cells were treated with 100 µM compound for 1 hour before the assay. As expected, clofilium tosylate was identified as an inhibitor, with an MTI = −0.71, nemisulide was identified as an uncoupler, MTI = 0.67, and azathioprine showed no significant mitochondrial toxic effects, MTI = −0.05, all similar to results using HepG2 cells.[2] Interestingly, pentamidine isethionate was identified as an inhibitor (MTI = −0.51) for primary hepatocytes, whereas it had been previously identified as a potential OxPhos inhibitor for HepG2 cells.[2]

Subsequently, dose response assays for each compound showing mitochondrial toxicity were performed (Figure 5B). Nimesulide showed an uncoupling effect, with an EC_{50} of ~33 µM, however, it should be noted that at higher concentrations (>50 µM, Figures 5B), inhibitory effects become evident, which is commonly observed for uncouplers. Clofilium tosylate and pentamidine isethionate showed exclusively inhibitory effects in the concentration range tested, with IC_{50} values of ~65 and 11 µM, respectively.

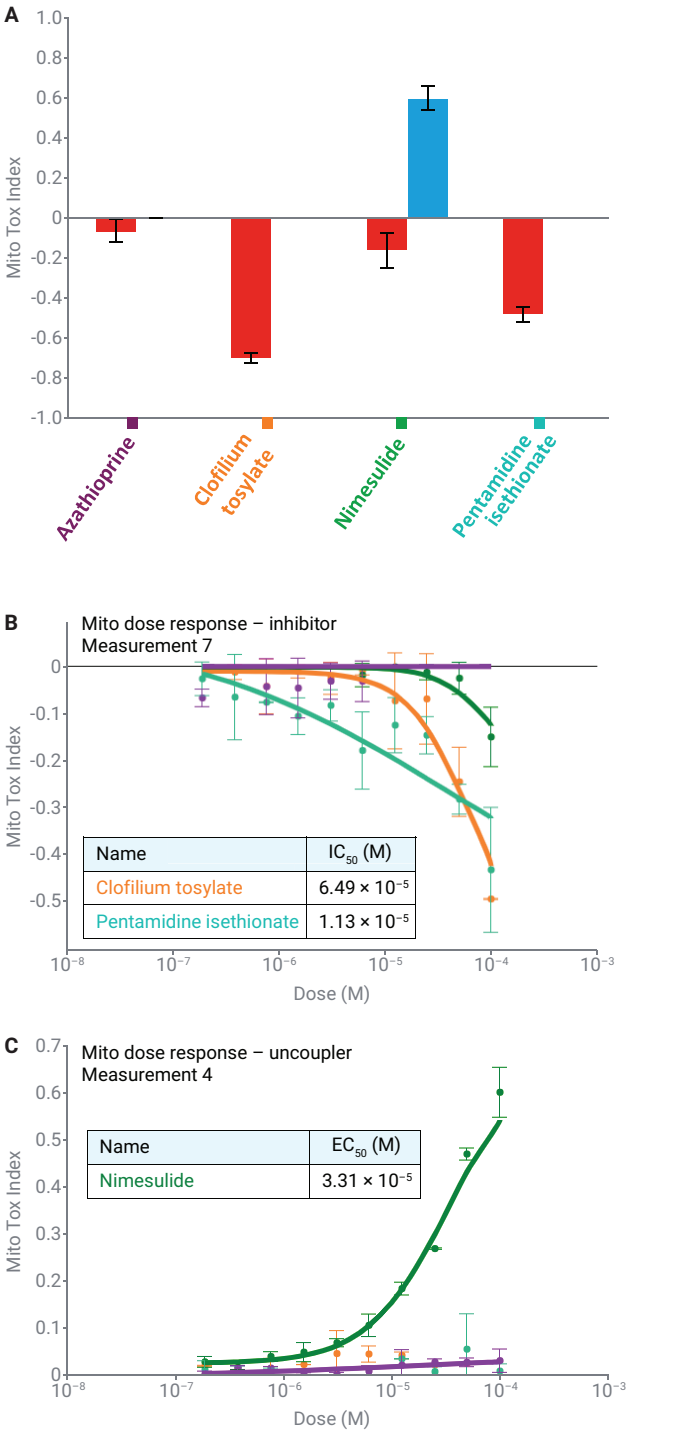

Figure 5. Agilent Seahorse XF Mito Tox assay using primary human hepatocytes. (A) Bar chart of Mito Tox index (MTI) values for azathioprine, clofilium tosylate, nimesulide, and pentamidine isethionate. Cells were treated with 100 µM of each compound for one hour prior to performing the XF Mito Tox assay. MTI values were calculated using Seahorse Analytics (mean ±S.D., n = 16). (B) Dose-response analysis of clofilium tosylate, nimesulide, and pentamidine isethionate. The cells were treated with the compounds for one hour at the concentrations indicated (0.2 to 100 µM). Dose-response curves, IC_{50}, and EC_{50} values were calculated using Seahorse Analytics. Mean ±S.D., n = 2.

Conclusion

Disruption of mitochondrial function can be detrimental, and is linked to drug-induced liver injuries, leading to drug attrition and post-market drug withdrawals. Direct measurement of mitochondrial oxygen consumption is a specific and sensitive indicator for the assessment of drug-induced mitochondrial toxicity.

Primary hepatocytes are the preferred sample material for *in vitro* prediction of DILI in drug safety tests. This application note outlines and demonstrates that the Agilent Seahorse XF Mito Tox assay can be used to identify and specifically distinguish between different modes of mitochondrial toxicity (i.e., uncoupling and inhibition) with high sensitivity, using primary human hepatocytes.

Of critical importance is the optimization of cell seeding density and FCCP concentration to ensure robust assay performance, and the sensitive, accurate detection of drug-induced mitochondrial toxicity. This application note provides a workflow that includes the optimization of cell density and FCCP concentration, as well as evaluation of data quality and assay performance, when qualifying a new lot of primary hepatocytes for use in the Seahorse XF Mito Tox assay.

While this application note describes and illustrates optimization and use of primary hepatocytes, this workflow is applicable to any new cell type to be used in the context of the Seahorse XF Mito Tox assay.

References

1. Principle of Mitochondrial Toxicity Assessment Using Agilent Seahorse XF Solution. *Agilent Technologies white paper*, publication number 5994-4732EN, **2022**.
2. A Customized XF Workflow for Detection and Characterization of Mitochondrial Toxicity. *Agilent Technologies application note*, publication number 5994-4778EN, **2022**.
3. Agilent Seahorse Mito Tox Assay Kit User Guide. *Agilent Technologies user guide*, publication number 5994-3715EN, **2022**.
4. Lonza, Document # BR-CryoHep-1 07/20.
5. Zhang *et al. J. Biomol. Screen*. **1999**, *4(2)*, 67–73. DOI:10.1177/108705719900400206

www.agilent.com

For Research Use Only. Not for use in diagnostic procedures.

RA45132.4569675926

This information is subject to change without notice.

Printed in the USA, August 8, 2023
5994-6476EN

Agilent
Trusted Answers