Application Note

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Metabolic Profiling Cancer Organoids Cultured in the Agilent Seahorse XF Flex Organoid Microplate

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Abstract

This document describes the application of the Agilent Seahorse XF Flex organoid workflow for metabolic profiling of cancer organoids using the Agilent Seahorse XF Flex analyzer and Agilent Seahorse XF Flex organoid microplate. The workflow was evaluated by examining the impact of metformin on mitochondrial function in cancer cell-derived organoids using the Agilent Seahorse XF 3D Mito Stress Test kit and Mito Tox assays. Key elements include optimizing organoid seeding procedures, image-based normalization, and the quantitative assessment of the metformin-induced mitochondrial dysfunction using the Mito Tox Index (MTI). This approach enables screening and characterization of drugs targeting mitochondrial function in three-dimensional (3D) culture models such as cancer organoids.

Introduction

Organoids are valuable tools in preclinical research, offering physiologically relevant three-dimensional (3D) models that recapitulate the architecture and function of native tissues better than traditional two-dimensional (2D) cell cultures. Drug screening in oncology typically begins with in vitro potency testing using 2D monolayer cultures, followed by validation in animal models such as xenografts. To improve clinical translatability, patient-derived xenograft (PDX) and humanized mouse models have been developed. However, the high cost, extended timelines, and ethical considerations associated with these models present significant limitations.

Patient-derived organoids (PDOs) have been proposed as a promising alternative for drug evaluation and personalized therapy design. PDOs retain key genetic and phenotypic features of the original tumors and enable high-throughput screening in a more physiologically relevant context. Supporting the shift, the U.S. Food and Drug Administration (FDA) introduced a roadmap aimed at reducing reliance on animal testing, emphasizing the development and validation of new approach methodologies (NAMs), including organoids and organ-on-a-chip systems.

Oxygen consumption rate (OCR) based analysis of mitochondrial respiration is a foundational technique in cancer metabolism research. Agilent Seahorse XF analyzers have been widely used for in vitro assessment of OCR and mitochondrial function, particularly in studies involving mitochondrial-targeting drugs and mitochondrial toxicity screening. The Agilent Seahorse XF Flex analyzer enables real-time measurement of cellular bioenergetics across many sample types, including organoids and tissue specimens. Specifically, the Agilent Seahorse XF Flex organoid microplate is optimized for matrix-embedded organoid culture, allowing long-term growth, high-resolution imaging, and comprehensive metabolic profiling.

This application note details the use of the Seahorse XF Flex organoid workflow to evaluate mitochondrial function in HCT116-H2B-GFP (HCT116) colon cancer cell-derived organoids embedded in Matrigel using XF Flex organoid microplates. Metformin (1, 1-dimethylbiguanide hydrochloride), a well-studied agent targeting tumor metabolism, was used to inhibit mitochondrial activity. This proof-of-concept study highlights the utility of Mito Tox Index (MTI)-based quantification for assessing drug effects on cancer organoids. The presented workflow may be readily adapted for PDO models in future applications.

Experimental

Materials and methods

Table 1. Material and equipment used in the Agilent Seahorse XF organoid workflow.

Product	Vendor	Part Number
Seahorse XF Flex analyzer	Agilent	S7851A or S7851AN
Seahorse XF assay medium		103866-100
Seahorse XF Flex organoid microplates		103865-100
Seahorse XF 3D Mito Stress Test kit		103016-100
Seahorse XF Cell Mito Stress Test kit		103015-100
Seahorse XF DMEM assay medium pack, pH 7.4		103680-100
BioTek Cytation 5 cell imaging multimode reader		
Matrigel matrix	Corning	356231
HCT116-H2B-GFP CRC cell line		
DMEM	Gibco	11995-065
Fetal bovine serum (FBS)		
Hoechst 33342	Thermo Fisher	62249
Metformin (1, 1-dimethylbiguanide hydrochloride)	Sigma	D150959

Organoid culture on XF Flex organoid microplate

HCT116-H2B-GFP (HCT116) cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS). To form organoids, 500 to 1,000 cells were embedded in 10 µL 50% Matrigel (~ 5 mg/mL protein) per well. The cells were cultured for five to seven days to reach an average diameter of ≥ 100 µm. To prevent cell adhesion beneath the Matrigel layer, a two-step seeding procedure was used. First, 6 µL ice-cold Matrigel was added and incubated at 37 °C for 20 to 30 minutes. Then, 4 µL cell suspension in ice-cold Matrigel was added on top and incubated for another 20 minutes. Finally, 250 µL prewarmed growth medium was added per well and refreshed every two to three days. For more detailed information, please see the Agilent technical overview.[1]

Seahorse XF Mito Stress Test

Since the optimal concentrations of oligomycin and rotenone/antimycin A (Rot/AA) were identified as 1.5 and 0.5 µM respectively in previous experiments,[1] the Agilent Seahorse XF Cell Mito Stress Test kit was used, which provides sufficient compound materials. Briefly, the cultured medium was replaced with 500 µL of prewarmed XF assay medium based on Seahorse XF DMEM medium, pH 7.4 supplemented with glucose, glutamine, and pyruvate. After washing once by replacing 450 µL of the assay medium, the plate was incubated at 37 °C for 45 minutes in a non-CO_2 incubator. The injection volume of the kit components was determined as described in the user manual of Seahorse XF Cell Mito Stress Test kit.[2]

Seahorse XF Mito Tox assay

Mitochondrial dysfunction by metformin was assessed using the Seahorse XF Mito Tox assay.[3] Organoids were treated overnight with varying concentrations of metformin. A vehicle control group and a Rot/AA control group should also be included. During the assay, oligomycin and FCCP were injected using the concentrations identified by Seahorse XF Mito Stress Test (see previous section). For more details about the principle and applications of this assay, see the Agilent Sesahorse Mito Tox assay white paper and application note.[4,5]

Image analysis and data normalization

Organoid imaging was performed using an Agilent BioTek Cytation 5 with 4x objectives. Brightfield and fluorescence images (Hoechst 33342 and GFP) were captured and z-projected. The total object area (Object Sum Area) or total fluorescence intensity integrated in the objects (Object Sum Int) was quantified using Gen5 software and used to normalize OCR data.

Data analysis

All data was uploaded to and analyzed in Agilent Seahorse Analytics using the companion views of the 3D Mito Stress Test and Mito Tox Screen. Three independent assay results executed using the identical assay template and instrument were combined using the Project feature in the software. Statistical comparison between 2D and organoid samples regarding to metformin MTI was done using GraphPad Prism (version 10.6.1).

Results and discussion

Optimizing the XF Mito Stress Test for HCT116 organoids

HCT116 cells were seeded at 1,000 cells per well using a two-step procedure, as detailed in the Experimental section. Briefly, cells were embedded in Matrigel and cultured for five to six days, forming multiple organoids per well. Representative z-projected images of organoids at days one, three, and six postseeding are shown in Figure 1, illustrating progressive growth and structural development.

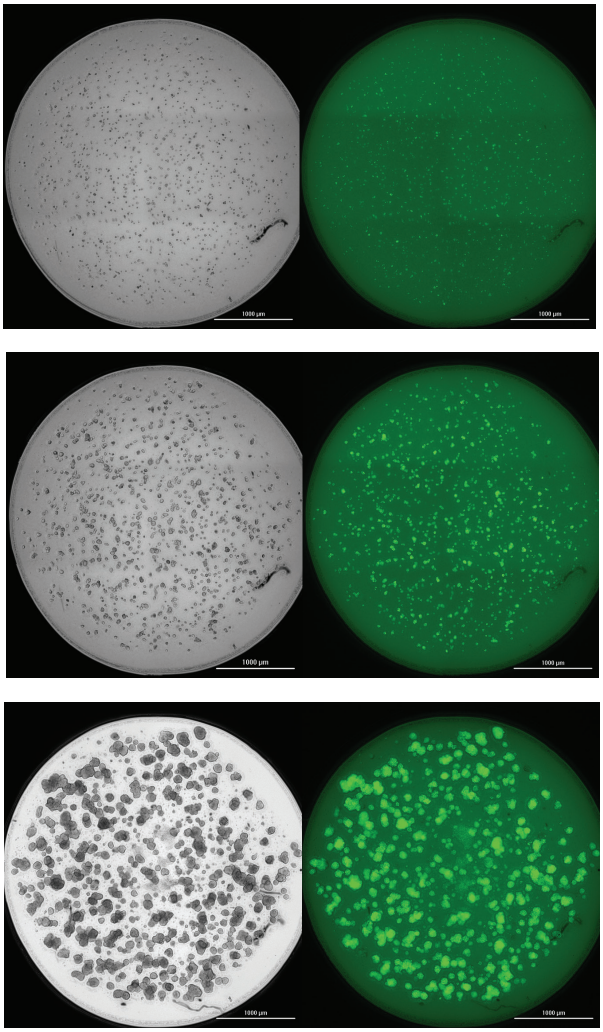

Figure 1. HCT116-H2B-GFP cancer organoid formation embedded in Matrigel within the Agilent Seahorse XF Flex organoid microplate. Brightfield (left) and GFP fluorescence images (right) were captured by an Agilent BioTek Cytation 5 cell imaging multimode reader using a Gen5 protocol that includes z-stacking with five slices over an 800 µm thickness (z-projected). Scale bar = 1, 000 µm.

Optimizing XF assay reagents, particularly carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), is critical, as the effective concentration can vary depending on the organoid type and source. For HCT116 organoids, the optimal concentrations of oligomycin and Rot/AA were consistent with those used for HCT116 cells cultured in 2D monolayers (data not shown). For FCCP optimization, a titration assay was conducted using five FCCP concentrations (0.25, 0.5, 1, 2, and 4 µM). It was determined 1 µM was optimal for inducing maximal respiration (Figure 2). Based on the optimization results, the subsequent experiments used the XF Cell Mito Stress Test kit instead of the XF 3D Mito Stress Test kit.

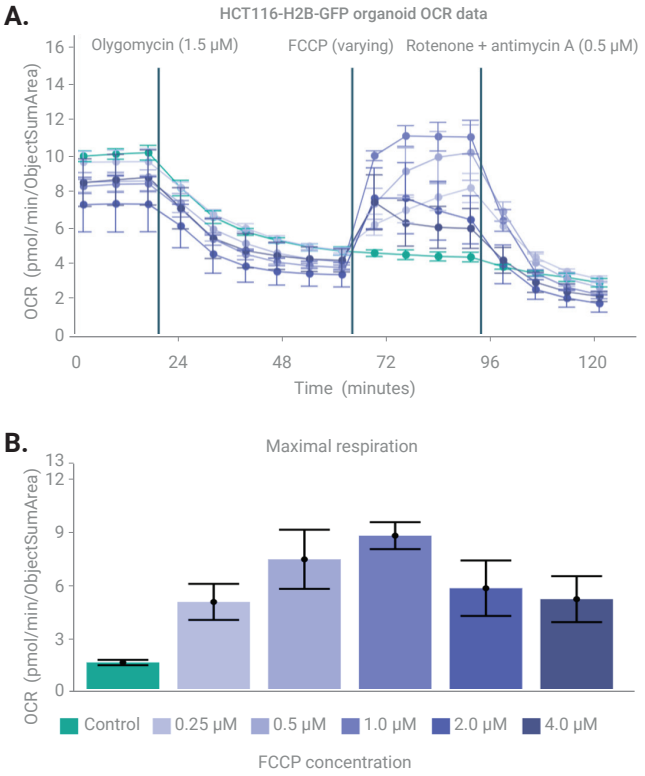

Figure 2. FCCP titration in HCT116 cell-derived organoid culture using the Agilent Seahorse XF Flex organoid microplate. HCT116 cells were seeded at 1,000 cells embedded in 11 µL of 50% Matrigel per well and then cultured for five days. A. OCR kinetic graph normalized by the brightfield total object area. B. Maximal respiration calculated by Agilent Seahorse Analytics software.

Metformin-induced mitochondrial dysfunction in cancer organoids

To assess the effect of metformin on mitochondrial function in the cancer organoid model, the XF Cell Mito Stress Test was performed on day five following overnight treatment with metformin at various concentrations. As shown in Figure 3, both basal and maximal OCR were significantly reduced in a dose-dependent manner, indicating that metformin impairs mitochondrial respiration in HCT116 organoids.

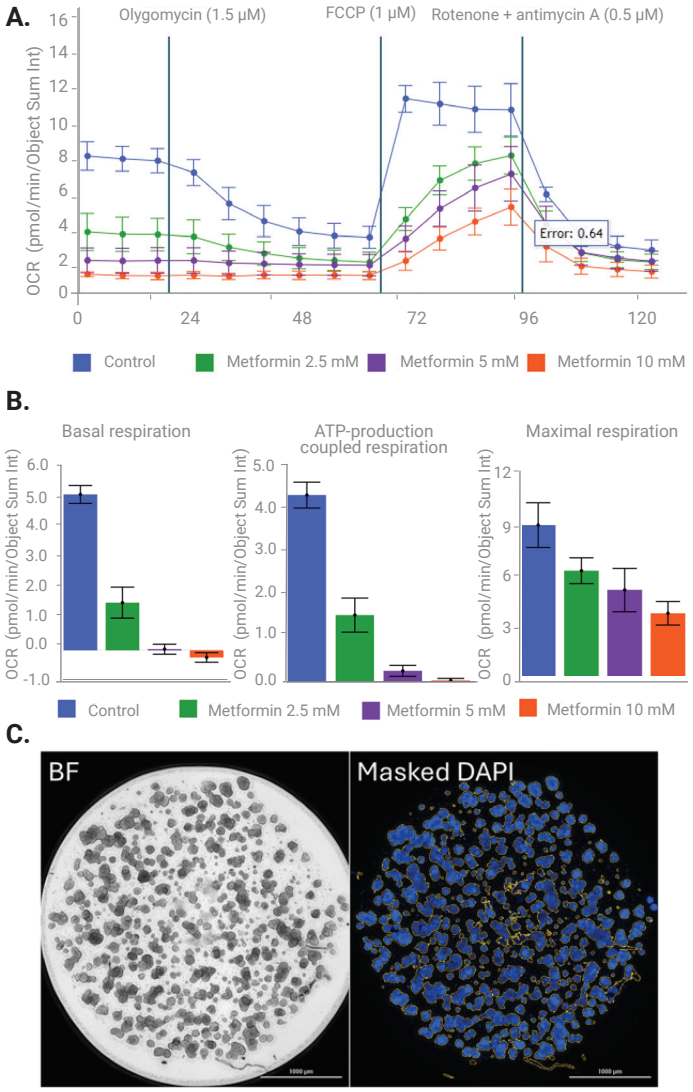

Figure 3. Metformin effect on mitochondrial respiration in HCT116 cancer organoids. HCT116-H2B-GFP cells were cultured in 50% Matrigel for five days. The organoids were cultured for another day in the presence of metformin (2.5, 5 or 10 mM) prior to performing the Agilent Seahorse XF Mito Stress Test. The nuclei were stained with Hoechst 33342 that was delivered at the last injection (rotenone/antimycin A mix in this case). A. OCR data changes in response to various doses of metformin. B. Changes in key bioenergetic parameters. C. Organoid images (4x) captured after the assay using the Agilent BioTek Cytation 5 cell imaging multimode reader for data normalization. The OCR data was normalized by the total integrated fluorescence intensity (Object Sum Int) of Hoechst 33342. Scale bar = 1, 000 µm.

Quantitative assessment of metformin-induced mitochondrial toxicity using the Mito Tox Index

While the XF Cell Mito Stress Test is a well-established and widely used tool for evaluating mitochondrial function—providing bioenergetic parameters like basal respiration, ATP production, and maximal respiratory capacity—it is not designed for rapid identification of compound impact on

mitochondrial function or mitochondrial toxicity. In contrast, the XF Mito Tox assay offers a more direct readout to assess mitochondrial function disruption or toxicity across different culture conditions and models (such as, 2D cells versus 3D organoids). By incorporating defined control conditions (vehicle and rotenone/antimycin A), the assay enables calculation of the MTI, a standardized metric for comparing dose-dependent mitochondrial inhibition or uncoupling. This targeted approach allows for more accurate and reproducible compound toxicity profiling, especially in drug screening and mechanistic studies.[4,5]

For this reason, the XF Mito Tox Assay was employed to assess the impact of metformin in HCT116 organoids. First, for the evaluation of assay robustness and sample suitability, Z'-factor analysis was performed, as shown in Figure 4. Z'-values for both uncoupler and inhibitor conditions exceeded 0.5, confirming that the assay is appropriate for screening drug-induced mitochondrial dysfunction in HCT116 organoid model. MTI values were then measured in organoids pretreated overnight with increasing concentrations of metformin. Accurate MTI calculation using Seahorse Analytics software requires two specific control groups: vehicle and rotenone/antimycin A (R/AA). Figure 5A presents the XF Mito Tox assay plate map used to examine the inhibitory effects of metformin, including four experimental groups with varying metformin concentrations alongside the two controls. Figure 5B shows representative data from the three independent assays. As shown, MTI values for metformin increased in a dose-dependent manner.

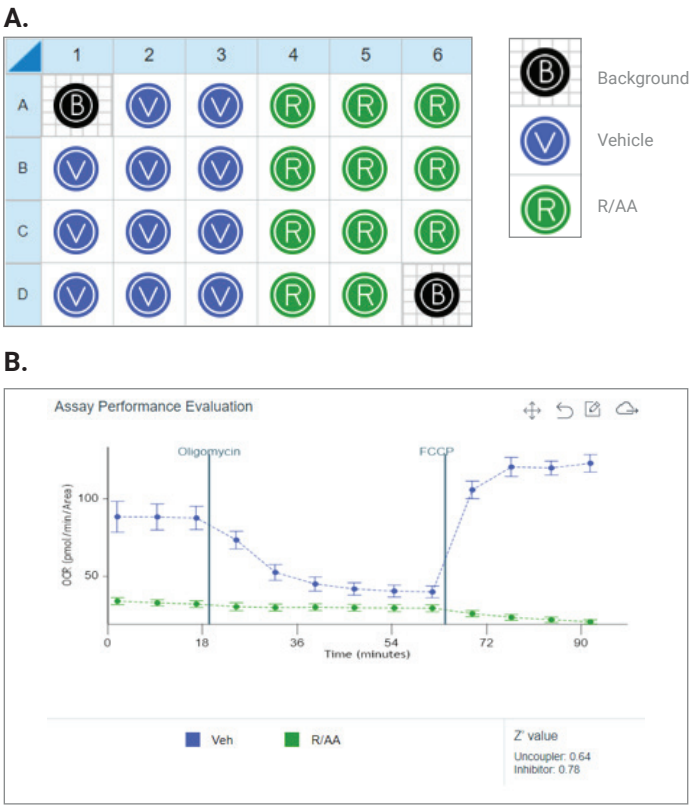

Figure 4. Assay performance evaluation by Z' value. The robustness of the Agilent Seahose XF Mito Tox assay using HCT116 organoids was evaluated by Z' calculation. A. Plate map for Z' evaluation. B. Z' evaluation result. All data were normalized by the organoid size (Object Sum Area) measured from images captured using the Agilent BioTek Cytation 5 cell imaging multimode reader.

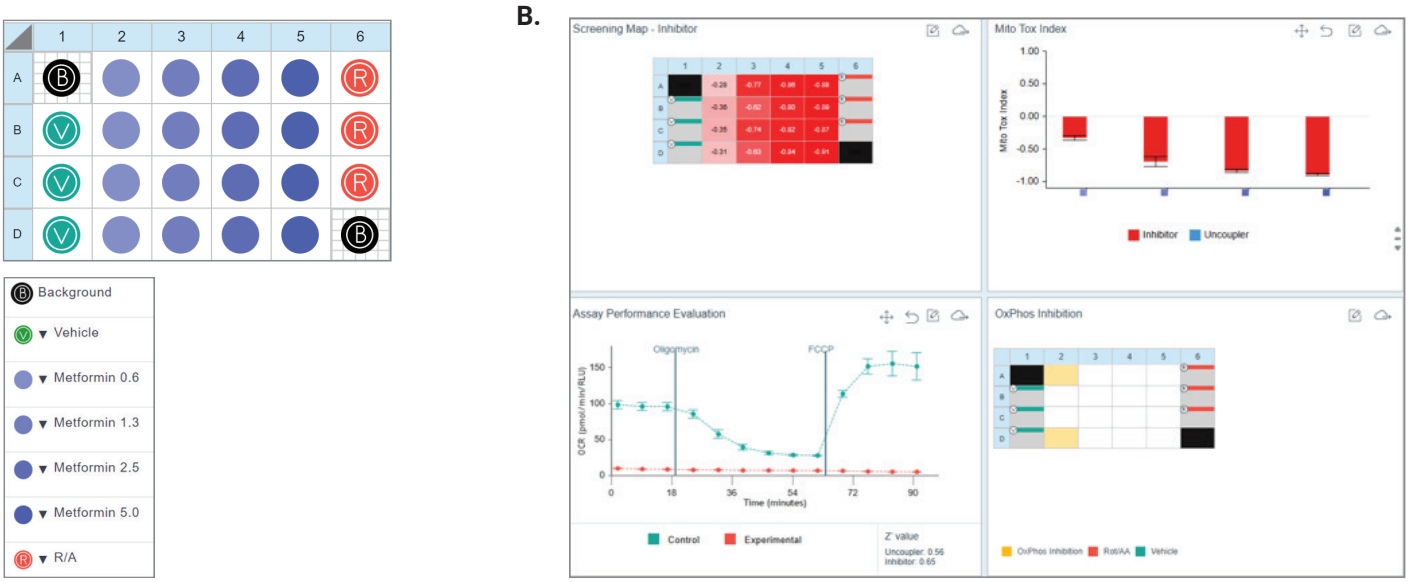

Figure 5. Agilent Seahorse XF Mito Tox assay results from metformin treated HCT116 organoids. A. Plate map of XF Mito Tox assay. The inhibitor MTI of metformin was measured after overnight exposure to metformin at four different doses (n = 4) as indicated. B. A representative data set from three repeated XF Mito Tox assays. The data displayed was obtained from Seahorse Analytics software. It includes inhibitor MTI heat map, Mito Tox Index bar chart, heat map for potential inhibitor of oxidative phosphorylation (OxPhos), and internal Z' value calculation (clockwise from the upper left panel).

Under the assay conditions, the IC_{50} of metformin was determined to be 2.05 mM in an independent set of XF Mito Tox assays (n = 3) using 2D-cultured HCT116 cells (data not shown). Notably, when using the HCT116 organoids, the MTI reached –0.5 at a lower concentration (1.3 mM), suggesting that organoids may exhibit greater sensitivity to metformin's inhibitory effects. To investigate this hypothesis, MTI values obtained from XF Mito Tox assays using both 2D cultures and 3D organoids were aggregated and compared. As shown in Figure 6, a two-way ANOVA revealed statistically significant differences in MTI values at the three higher metformin concentrations (1.3, 2.5, and 5 mM) between the two culture formats (2D versus organoid). These results indicate a consistent and dose-dependent difference in metformin sensitivity between 2D and organoid models.

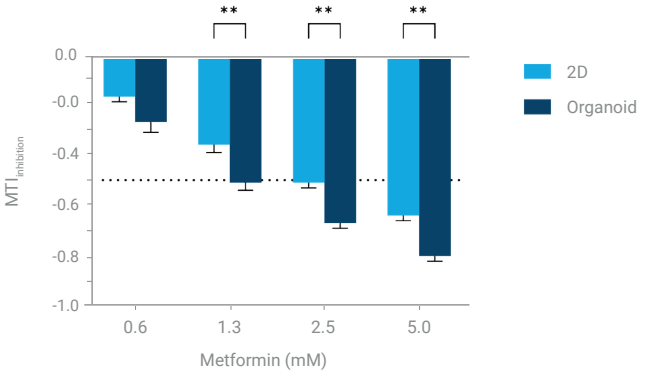

Figure 6. Comparison of mitochondrial inhibition by metformin between HCT116 cells cultured in 2D and in 3D organoids. The inhibitory MTI of metformin was assessed from 2D cultured HCT116 and organoids after overnight exposure by the Agilent Seahorse XF Pro and Agilent Seahorse XF Flex respectively. The values presented by means ± S.E.M. of three independent assays for each culture condition performed on different days, which were calculated by using the Project feature in Seahorse Analytics software. The significance of difference was analyzed by two-way ANOVA using GraphPad Prism software.

Conclusion

This application note demonstrates the use of a robust workflow for metabolic profiling of cancer organoids and the assessment of drug impact on mitochondrial function, using the Agilent Seahorse XF Flex analyzer and Agilent Seahorse XF Flex organoid microplate. The enhanced sensitivity of organoids to metformin underscores the importance of 3D models in drug screening and mechanistic studies. This approach is adaptable to various organoid types and supports the development of NAMs for preclinical research.

References

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5. Kam, Y.; Rogers, G.W.; Winer, L.; Schwalfenberg, M.; Romero, N. A Customized XF Workflow for Detection and Characterization of Mitochondrial Toxicity. *Agilent Technologies application note,* 5994-4778EN, **2022**.

Products used in this application

Agilent products

Agilent Seahorse XF Flex analyzer

Agilent Seahorse XF Flex organoid FluxPak

Agilent Seahorse XF 3D Mito Stress Test kit

Agilent Seahorse XF Cell Mito Stress Test kit

Agilent Seahorse XF DMEM assay medium pack

Agilent BioTek Cytation imaging multimode reader

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