Technical Overview

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Evaluation of Edge Effects and Evaporation from Agilent Seahorse XF Pro M Cell Culture Plates

Introduction

Agilent Seahorse XF technology simultaneously interrogates the two main metabolic pathways, mitochondrial respiration and glycolysis, for live cells in real time, to provide functional kinetic measurements of cellular bioenergetics. This technology provides the flexibility for researchers to customize experiments to target specific research questions, across a range of application areas.

Although the 96-well platform allows a variety of parameters to be tested during a single assay, a common challenge to overcome during multiwell cellular assays using this format is edge effect. The Agilent Seahorse XF Pro M cell culture microplate was designed to mitigate edge effects and their impact on XF data to provide a superior level of confidence when performing XF assays.

The term "edge effect" is used to describe a common result obtained during microplate cellular assays. Differences in cell growth and behavior are often observed in cell plates containing 96 or more wells. These differences are found in the periphery wells of these microplates and can impact assay results. If severe enough, some researchers may choose not to use the periphery wells for their samples, and instead create a buffer region to absorb the environmental effects that can lead to the observed differences. These environmental effects are often due to differences in humidity, temperature, or evaporation.

The XF Pro M cell culture plate was designed to mitigate edge effects so that all 96 wells behave similarly. As shown in Figure 1, a series of moats were added to the periphery of the microplate. By filling these sections with water, a temperature and evaporation barrier can prevent these edge effects from impacting the outermost wells.

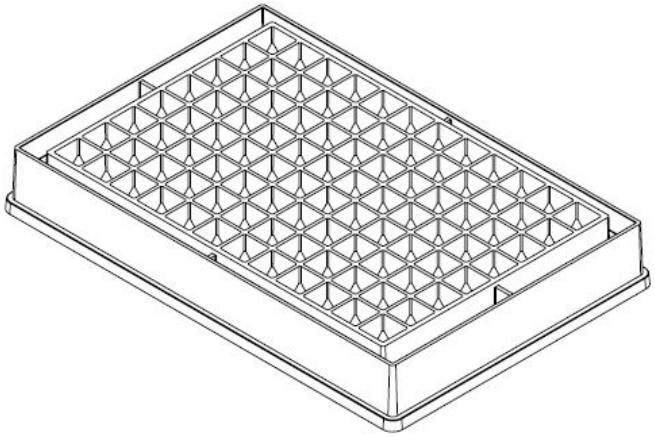

Figure 1. Illustration of an Agilent Seahorse XF Pro M cell culture microplate, showing the moat wells around the well array.

The Agilent Seahorse XF Pro analyzer features an advanced temperature control system designed to reduce temperature gradients between outer and inner wells. The design includes a custom edge-well tray heater and a manifold heater that covers the consumable when performing an assay. These features create a small, temperature-controlled environment to ensure optimal XF assay performance.

The purpose of this technical overview is to demonstrate the performance of the XF Pro M cell culture microplates. Both rate-based edge effects and evaporation results are described in the following sections. Evaporation rate can impact cell growth while rate differences between inner and outer wells are used to quantify edge effects on the XF Pro analyzer.

Materials and methods

Edge effect experiments

Background: The evaluation of the XF Pro M cell culture plates was performed using an XF Pro analyzer with two adherent cell lines, C2C12 and HepG2 (ATCC). Adherent cell types are most affected by edge effects due to their preparation requirements, and are therefore suitable for use in such evaluations.

Note: When planning an XF assay, Agilent recommends characterizing the cell seeding density and optimal reagent concentrations before starting the assay. Please contact Agilent Technical Support with any questions.

Cell culture: All cell lines were cultured as specified by the supplier. Twenty-four hours before the assay, the cells were counted and seeded at a predetermined optimal density in XF Pro M cell culture microplates (part number 103775-100). Five plates were seeded per cell type. One cell density was used for all wells, except for the four corners, which were used for background correction, see the plate layout in Figure 2.

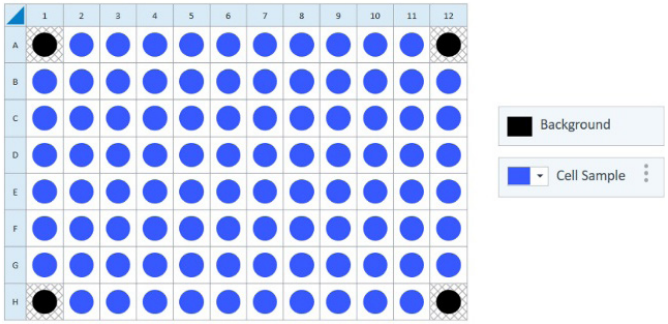

Figure 2. Experimental layout of the Agilent Seahorse XF Pro M cell culture plate. Blue indicates cell sample and black indicates background.

Assay procedure: The standard XF Cell Mito Stress Test was performed using the Agilent Seahorse XF Cell Mito Stress Test kit (part number 103015-100), following the protocols provided in the kit user manual.

Data analysis: Assay results were used to calculate edge effects. The inner 60 wells were grouped as "inner" wells, and the outer 32 were grouped as "outer" wells. Basal rates (rate measurement 3) were used for analysis. Both OCR and PER edge effects were calculated, using Equation 1.

Equation 1. Edge effect calculation.

$$\text{Edge Effect (\%)} = \left| \frac{\text{Average}_{\text{inner}} - \text{Average}_{\text{outer}}}{\text{Average}_{\text{inner}}} \right| \times 100$$

Evaporation experiments

Background: The evaporation from the XF Pro M cell culture microplate was evaluated to establish the overall evaporation amount and the uniformity of evaporation by measuring the water volume in each well (see "Measuring water volume" for method description). Test plates with equal starting water volumes were placed in a XF Pro analyzer, and subjected to the process for a typical XF assay. Plates were then read in a plate reader and water volumes were determined using a calibration curve.

Measuring water volume: To calculate the volume of water in each well of the XF Pro M cell culture plate, the optical density of water was measured at 977 nm and 900 nm in a plate reader. Water exhibits a peak in absorbance at 977 nm while the reading at 900 nm serves as a baseline. The difference of these two measurements is directly proportional to the pathlength of the sample in the well. Pathlength was correlated to volume and a calibration curve was used to determine the volume of water in each well. Plates and reagents were always prewarmed to 37 °C before measurement.

Calibration curve: A calibration curve was established by measuring the absorbance of water samples with known volumes on an XF Pro M cell culture plate. Three plate replicates were measured and volumes ranging from 50 to 250 µL were included. These data were then used to create a calibration curve to calculate the water volume in each well for test plates.

Test procedure: Agilent Seahorse XFe96 cartridges were hydrated, and empty XF Pro M cell culture plates and water was warmed in a 37 °C incubator overnight before running the XF assay. After the cartridge was calibrated, before loading the test plate, 200 µL of water was added to each well of the plate and 1 mL was added to each section of the moat. The XF assay was run for six hours to demonstrate the worst-case evaporation. Measurements from the XF Pro analyzer were collected every 15 minutes. Immediately upon assay completion, absorption measurements were taken at 977 and 900 nm on a Tecan plate reader.

Data analysis: Using the calibration curve that was created the volume remaining in each sample well after the XF assay was calculated. The percentage of volume lost was calculated for each well and the average loss per plate was found.

Results and discussion

Evaporation

Evaporation throughout a six-hour XF assay was evaluated by the method that measures the water volume changes, as described previously. Excessive evaporation may impact overall cell performance due to changes in the concentration of various components. In this assay, a simulated six-hour XF assay was performed in an XF Pro M cell culture plate filled with water to demonstrate the amount of evaporation during the assay. Note that a typical XF assay would be run with a medium containing various components that may affect evaporation rates. Therefore, these results serve as a guideline, but results may vary.

The graph in Figure 3 shows average evaporation across a 96-well plate after six hours in an XF Pro analyzer. The graph shows the average value for each position over six different plates. The average evaporation across all wells of all six plates is 7.99%. The edge wells do evaporate at a faster rate, but no single well exceeded 20% evaporation in all test runs.

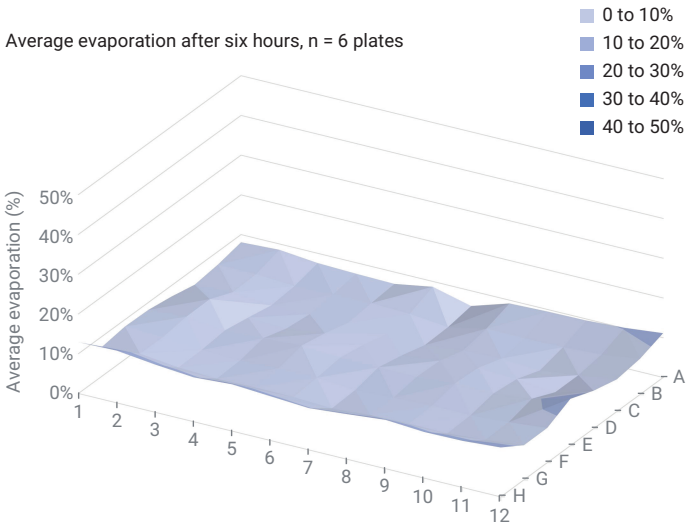

Figure 3. The average evaporation was measured across a 96-well plate after a six-hour incubation in an Agilent Seahorse XF Pro analyzer.

Edge effects

Edge effects that occurred during an overnight cell-growth stage were quantified for oxygen consumption rate (OCR) and proton efflux rate (PER) measurements using the XF Cell Mito Stress Test on the XF Pro analyzer.

While all 92 wells of every plate were prepared and treated in the same manner, for this analysis, the 32 wells on the periphery of the plate were re-assigned as an "outer wells" group, while the inner 60 wells were re-assigned as "inner wells". The average rate and standard deviation of each group were calculated for every third measurement (measurements 3, 6, 9, and 12 of an XF Cell Mito Stress Test), representing the basal measurement and measurements after oligomycin, FCCP, and Rotenone/antimycin A injection (Figure 4). The results showed comparable signals between the outer well group and the inner well group for all measurements.

For each plate, the edge effect was then quantified for these measurements using Equation 1. The results presented in Tables 1 (OCR) and 2 (PER) show that edge effect for all measurements is below 10% in all test plates. These experiments demonstrated that very little edge effect can be achieved throughout the course of an assay.

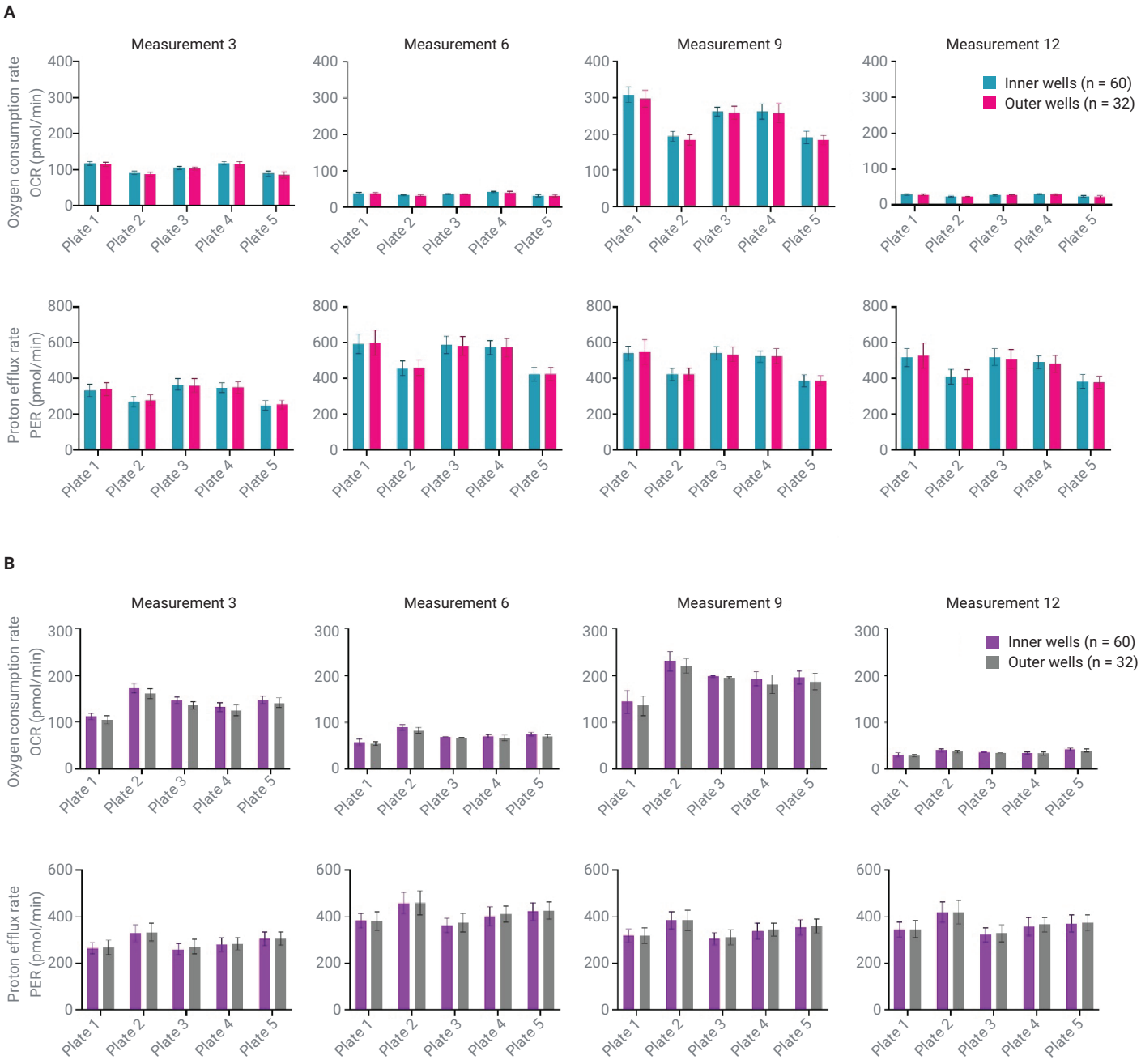

Figure 4. The average and standard deviation of the inner 60 wells (blue or purple) and outer 32 wells (pink or grey) of each plate, at every third measurement is shown. (A) Shows the data for five plates containing C2C12 cells, while (B) shows the data for five plates containing HepG2 cells. Each measurement corresponds to a different segment of the Mito Stress Test Assay. Basal measurements, oligomycin treatment, FCCP treatment, and rotenone/antimycin A treatment corresponds to measurements 3, 6, 9, and 12, respectively.

Table 1. Edge effect for OCR measurements.

OCR Measurement	C2C12				HepG2			
	3	6	9	12	3	6	9	12
Plate 1	2.09%	2.07%	3.95%	2.49%	6.62%	5.98%	5.83%	6.30%
Plate 2	3.82%	3.83%	4.78%	1.79%	6.73%	7.03%	4.28%	8.31%
Plate 3	1.04%	0.14%	1.38%	0.81%	7.73%	2.84%	1.42%	3.76%
Plate 4	2.82%	3.30%	1.49%	2.82%	5.62%	6.13%	5.89%	1.15%
Plate 5	4.79%	4.44%	3.65%	7.13%	4.59%	6.16%	4.93%	5.61%

Table 2. Edge effect for PER measurements.

PER Measurement	C2C12				HepG2			
	3	6	9	12	3	6	9	12
Plate 1	2.20%	1.24%	0.74%	1.84%	1.26%	0.09%	0.42%	0.52%
Plate 2	2.93%	0.57%	0.01%	0.64%	1.29%	0.33%	0.10%	0.42%
Plate 3	1.37%	1.18%	1.12%	2.16%	3.96%	3.00%	2.29%	1.65%
Plate 4	0.70%	0.47%	0.31%	1.52%	2.11%	2.10%	2.24%	2.37%
Plate 5	2.04%	0.05%	0.13%	1.21%	0.47%	0.89%	1.48%	1.03%

Conclusion

The Agilent Seahorse XF Pro M cell culture microplate, when used with an Agilent Seahorse XF Pro analyzer, provides an extra level of confidence when measuring extracellular flux across all 96 wells. The addition of the peripheral reservoir can aid in mitigating the impact of environmental factors on the outer wells of the plate. While all edge effects may not be completely eliminated, the use of these products with ideal sample preparation may result in excellent assay performance. The Agilent Seahorse XF Pro M cell culture microplate provides researchers with an extra layer of confidence, particularly when using the outer wells of the assay plate.

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