Application Brief

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Validation of a Six-Hour Agilent Seahorse XF Assay on the Agilent Seahorse XF Pro Analyzer

Introduction

Agilent Seahorse XF technology allows researchers to simultaneously measure oxidative phosphorylation and glycolysis from live cell samples in real time. Although many Agilent Seahorse XF assay kits have run times of less than two hours, Agilent has validated the use and performance of the Agilent Seahorse XF Pro Analyzer for a run time of up to six hours. This validation allows longer-term metabolic analysis with greater confidence in assay performance.

Agilent Seahorse XF assays can be performed for a finite period due to several factors, which include: (1) samples are assayed in atmospheric conditions with minimal buffering bicarbonate, which may cause pH decreases after long periods; (2) a single Seahorse XF assay must be run continuously in a system, which is not immune to evaporation. Significant evaporation can cause metabolic changes such as increases in glycolysis[1] to occur, affecting results and experimental interpretation; (3) the collection, calculation, and storage of large amounts of data can cause computing errors and unforeseen system crashes.

This application brief explains the validation of a six-hour Seahorse XF assay. The previously mentioned factors were each considered to validate the performance of this assay. This validation was accomplished using the XF Pro Analyzer featuring advanced temperature control with Agilent Seahorse XF Pro M cell culture microplates and the Agilent Seahorse XF Cell Mito Stress Test kit.

Experimental

Materials

Agilent Seahorse XF Pro M cell culture microplates with Agilent Seahorse XF96 Extracellular Flux assay kits (part number 103775-100), Agilent Seahorse XF Cell Mito Stress Test kits (part number 103015-100), Agilent Seahorse XF DMEM assay medium, pH 7.4 (part number 103680-100) , Agilent Seahorse XF 1.0 M glucose solution (part number 103577-100), Agilent Seahorse XF 100 mM pyruvate solution (part number 103578-100), and Agilent Seahorse XF 200 mM glutamine solution (part number 103579-100). A549 cells were purchased from the American Type Culture Collection. All other materials were purchased from Sigma-Aldrich.

Sample preparation

A549 cells were recovered from frozen stock and were seeded at 1.5×10^4 cells/well in an XF Pro M cell culture microplate. Ninety-two wells were seeded with cells in DMEM medium supplemented with 10% fetal bovine serum (FBS). Each section of the periphery moat was filled with 1.0 mL of cell culture-grade sterile water. This plate was left at room temperature in a BioSafety cabinet for one hour before incubating in a 37 °C, 5% CO_2 incubator for overnight growth and proliferation.

An XF96 sensor cartridge was prepared by adding 200 µL of XF calibrant to each well of the utility plate, assembled with a hydrobooster and sensor cartridge before incubating in a 37 °C, non-CO_2 incubator overnight.

The next day, XF DMEM media, pH 7.4 was supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose using XF reagents, and warmed to 37 °C. This medium was used to wash the plate containing A549 cells twice before placing the plate in a non-CO_2 incubator for 60 minutes. While the plate was incubating, the same medium was used to prepare 10 µM oligomycin, 10 µM FCCP, and 5 µM antimycin A/rotenone solutions from the XF MST kit reagents.

The sensor cartridge was obtained after overnight hydration and the hydrobooster was removed before loading the drug ports. Port A was loaded with 25 µL of the oligomycin solution, Port B was loaded with 25 µL of the FCCP solution, Port C was also loaded with 25 µL of the FCCP solution, and Port D was loaded with 25 µL of the antimycin A/rotenone solution. Then, the cartridge and cell plate were used for XF analysis on the Agilent Seahorse XF Pro Analyzer.

XF instrumentation

Agilent Seahorse XF Pro Analyzer with Wave Pro controller software

Live cell metabolic measurements were collected for a total duration of six hours on the Seahorse XF Pro Analyzer. The plate map included one sample group containing 92 sample wells, and the four corner wells were used for background correction. The default measurement protocol for 96-well XF assays is a three-minute mix followed by a three-minute measurement. In this case, rate data were collected once every 15 minutes adhering to the following assay protocol:

1. **User Action:** Load XF96 sensor cartridge
2. Cartridge Calibration
3. **User Action:** Switch utility plate to cell plate
4. Equilibration
5. **Baseline Measurements:** five cycles
    a. 3:00 mix
    b. 9:00 wait
    c. 3:00 measure
6. Inject Port A (oligomycin)
7. **Measurements:** five cycles
    a. 3:00 mix
    b. 9:00 wait
    c. 3:00 measure
8. Inject Port B (FCCP)
9. **Measurements:** four cycles
    a. 3:00 mix
    b. 9:00 wait
    c. 3:00 measure
10. Inject Port C (FCCP)
11. **Measurements:** four cycles
    a. 3:00 mix
    b. 9:00 wait
    c. 3:00 measure
12. Inject Port D (antimycin A/rotenone)
13. **Measurements:** six cycles
    a. 3:00 mix
    b. 9:00 wait
    c. 3:00 measure

Data analysis

Upon assay completion, the raw data were saved and exported for analysis in Wave Pro desktop software. Initial data quality checks were performed using the data quality analysis view, to automatically identify outlier wells and injection irregularities. Oxygen consumption rate (OCR) and proton efflux rate (PER) data were analyzed for basal measurement coefficient of variation (CVs) and edge effects.

Results and discussion

Although typical Seahorse XF assay kits have run time of less than two hours, longer assay times are warranted. Factors such as medium buffering, evaporation, and computing ability can limit assay duration. The Seahorse XF Pro Analyzer and Seahorse XF Pro M cell culture microplates allow a validated performance level during an assay six hours in duration.

The kinetic results for a six-hour modified XF Cell Mito Stress Test are shown in Figure 1. No statistical outliers or problems related to performing real-time injections were identified. The last measurement (measurement 24) ended at minute 360, and the total measurement time shown is six hours.

Basic assay performance metrics were collected during the assay run, including the coefficient of variation (%CV) during the basal measurements. In this experiment, measurement five was used to evaluate basal performance, as it was the last measurement taken before injection. As shown in Table 1, the basal OCR and PER % CV are 5.26 and 8.74%, respectively. These values are well within the expected variability performance for a Seahorse XF Pro assay.

Because in many multiwell, live cell assays, edge effect is a common concern, the difference between the inner and outer wells was also analyzed at different points during the assay. The following formulas were used to calculate OCR and PER edge effects for the assay plate, which contained one group over all 92 sample wells.

$$\text{OCR Edge Effect (\%)} = \frac{\text{Avg OCR}_{\text{Inner}} - \text{Avg OCR}_{\text{Outer}}}{\text{Avg OCR}_{\text{Inner}}}$$

$$\text{PER Edge Effect (\%)} = \frac{\text{Avg PER}_{\text{Inner}} - \text{Avg PER}_{\text{Outer}}}{\text{Avg PER}_{\text{Inner}}}$$

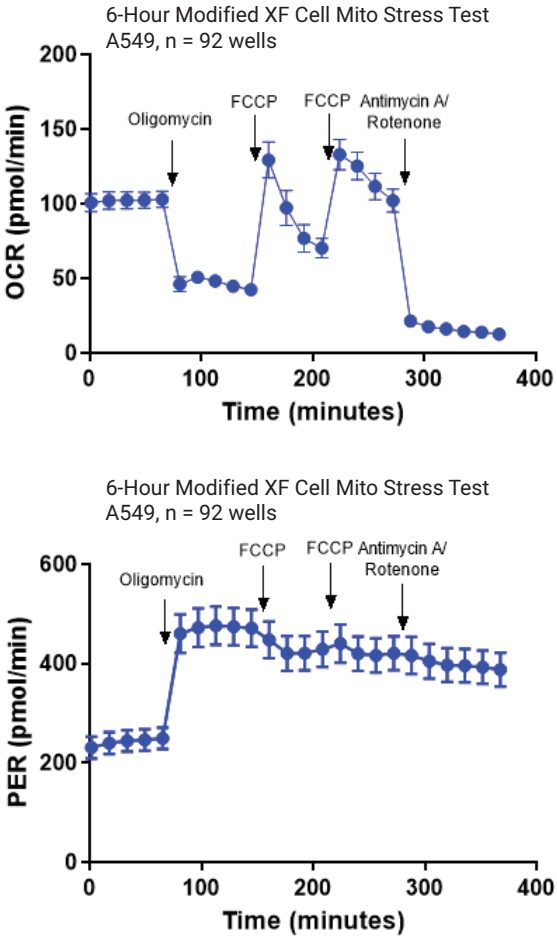

Figure 1. OCR and PER kinetic traces of a six-hour modified Agilent Seahorse XF Cell Mito Stress Test on A549 cells performed on an Agilent Seahorse XF Pro Analyzer.

Table 1. Measurement statistics for basal OCR and PER taken at measurement five of a modified Agilent Seahorse XF Cell Mito Stress Test on an Agilent Seahorse XF Pro Analyzer.

	OCR (pmol/min)	PER (pmol/min)
Average Rate	103.11	249.94
Standard Deviation	5.42	21.84
Coefficient of Variation	5.26%	8.74%

Rate differences between inner and outer wells were calculated at the last basal measurement (measurement number 5), at the last measurement after oligomycin injection (10), the first measurement after each FCCP measurement (11 and 15), and the last measurement after antimycin A/rotenone injection (24). The calculated edge effect was under 10 % over the entirety of the assay in both the OCR and the PER channels, as presented in Table 2.

Conclusion

The Agilent Seahorse XF Pro Analyzer and the compatible Agilent Seahorse XF Pro M cell culture microplates enable consistent and robust Seahorse XF assay data to be collected from start to finish for six hours. This protocol has been validated to meet the specifications for a six-hour assay shown in Table 3. This effort was intended to focus on the following sources of error or variability:

- **Evaporation from sample wells:** The configuration of the Seahorse XF Pro Analyzer and the XF Pro M cell culture plate were designed to mitigate evaporation during an XF assay.
- **Drug port evaporation:** Evaporation from cartridge compound ports was a consideration for the validation of this assay. Testing confirmed that evaporation did not hinder the injections during this assay.
- **Performance degradation:** A key specification of this assay was to ensure that the performance did not degrade throughout the experiment. As shown, performance was maintained throughout the assay.

Table 2. Calculated OCR and PER edge effects over the duration of a six-hour modified Agilent Seahorse XF Cell Mito Stress test performed on XF Pro M cell culture microplates with an Agilent Seahorse XF Pro Analyzer.

Measurement	OCR Edge Effect	PER Edge Effect
5	2.75%	1.15%
10	3.73%	3.80%
11	8.54%	3.89%
15	5.58%	5.41%
24	0.96%	6.58%

Table 3. Performance specifications for a six-hour XF assay using XF Pro M cell culture microplates on Agilent Seahorse XF Pro analyzer.

Specification	Limit
OCR Coefficient of Variation (Mid-Rates)	≤20%
PER Coefficient of Variation (Mid-Rates)	≤30%
Sample Well Evaporation	≤25%
Average Evaporation (All Wells)	≤10%
OCR Edge Effect	≤10%
PER Edge Effect	≤10%

Reference

1. Ozturk, S. S.; Palsson, B. O. Effect of Medium Osmolarity on Hybridoma Growth, Metabolism, and Antibody Production. *Biotechnology and bioengineering* **1991**, *37(10)*, 989–993.

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RA44686.637337963

Printed in the USA, May 13, 2022
5994-4696EN

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