Technical Overview

Cell Metabolism

Agilent

Trusted Answers

Improving Real-Time Metabolic Analysis in Islets and Spheroids Using the Agilent Seahorse XF Flex 3D Capture Microplate-S

Introduction

Agilent Seahorse XF technology is an integrated label-free platform that delivers real-time functional metabolic analysis, providing key parameters, such as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which are vital indicators of mitochondrial health, toxicity, glycolysis, and overall cellular (dys)function. The Agilent Seahorse XF Flex analyzer features improved sensitivity and precision, a broad detection range, and dedicated consumables designed for optimized workflows compatible with three-dimensional (3D) study models, such as tissue, organoids and coculture materials.

In this technical overview, we present an optimized workflow for analyzing pancreatic islet metabolism with enhanced sensitivity, and reduced sample requirements using the Seahorse XF Flex analyzer and the Agilent Seahorse XF Flex 3D capture microplate-S. This workflow provides data on key energy pathways that drive insulin secretion and overall islet health, allowing researchers to gain valuable insight into glucose responsiveness, mitochondrial efficiencies, and functional heterogeneity across islet populations. These data can help to identify dysfunctional islets, optimize culture conditions, and evaluate therapeutic interventions, thus advancing diabetes research, improving islet transplantation outcomes, and developing targeted therapies. Additionally, this workflow is compatible with small, multi-spheroid samples, making it suitable for broader applications in 3D metabolic analysis.

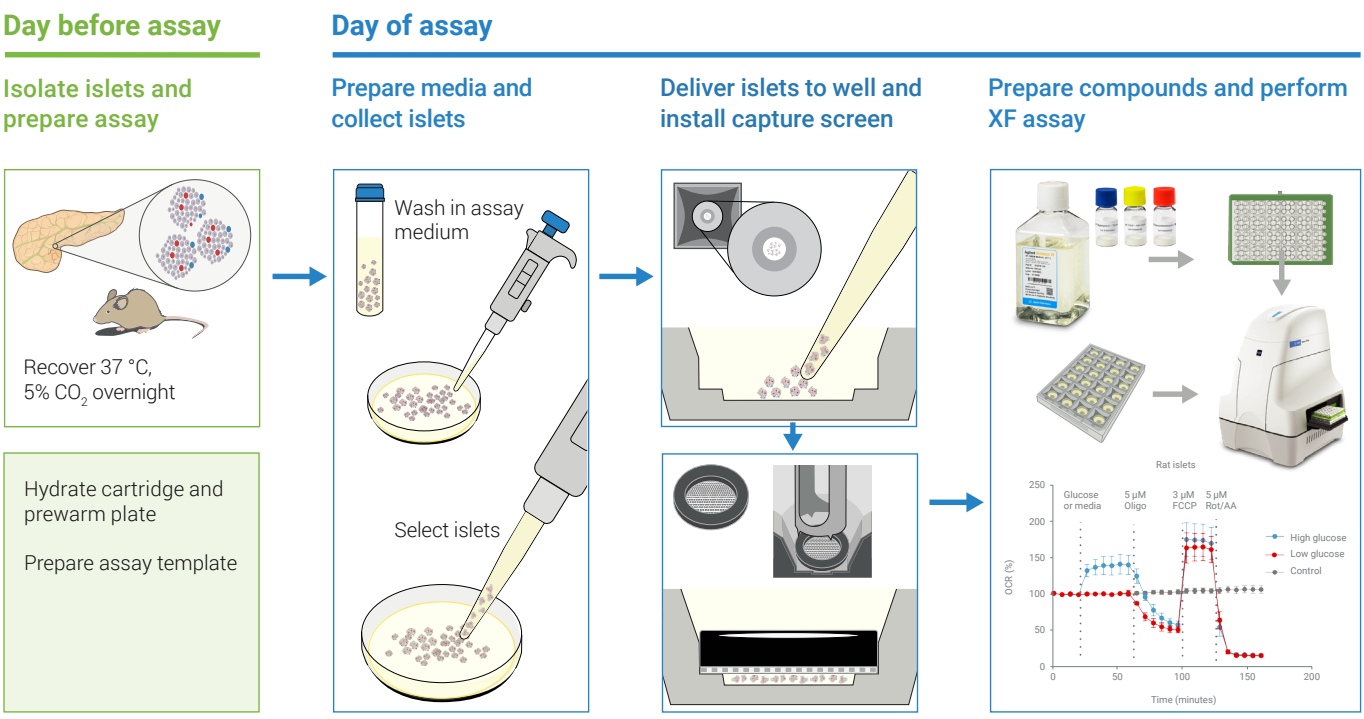

Figure 1. Pancreatic Islet workflow using Agilent Seahorse XF 3D capture microplate-S. Pancreatic islets are delivered to XF 3D capture microplate-S sample chambers and are held in place with uniquely designed capture screens optimized for small 3D samples.

Islet XF assay workflow

The assay workflow illustrated in Figure 1 outlines the key steps in performing the Agilent Seahorse XF assay with islet samples, including sample preparation, assay plate assembly, and materials needed. The following subsections provide the details for each of the steps involved. This workflow can be modified, if needed, to meet your intended research goals.

Assay procedures

Day prior to assay

Hydrate cartridges and place in 37 °C non-CO_2 incubator. Place the XF 3D capture microplate-S and screens in a 37 °C non-CO_2 incubator to degas the plastics overnight.

Isolation of pancreatic islet

Prepare whole islets following the protocol(s) used in your laboratory and recover overnight under standard conditions for islet culture.[1–3] The islets used in this study were purchased from Prodo Laboratories or Joslin Diabetes Center isolated from five male Sprague Dawley rats, 8 to 12 weeks of age. Islets from Prodo labs were pooled and recovered for 36 hours and shipped overnight at 6 to 10 °C in PIMT media (Prodo Laboratories). Upon arrival, islets were washed with PIM(R) medium, centrifuged, and resuspended at 2,500 IEQ per 10 mL of complete PIM(R). Islets from Joslin Diabetes Center were transported on the same day of isolation in RPMI 1640 with glutamine, 10% fetal calf serum (FCS) and 1% penicillin/streptomycin on ice and placed in the same media at 100 islets per mL. Islets were recovered overnight at 37 °C 5% CO_2 in nontissue culture treated petri dishes before performing XF assays.

Preparation of tumor spheroids

HCT116-H2B-GFP cells were maintained in Advanced DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin/streptomycin antibiotics. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and routinely passaged at 80% confluency. HCT116-H2B-GFP spheroids were prepared by plating 200 cells per cavity in 6-well Elplasia round-bottom plates (Corning). Spheroids were grown for 6 to 7 days to reach the size of ~ 150 µm in diameter before being used in assays.

Day of assay: Islet workflow

Prepare islet XF assay medium

Prepare 100 mL Agilent Seahorse XF DMEM pH 7.4 assay medium supplemented with 2.5 mM glucose, 2 mM glutamine, and 1 mM pyruvate according to Table 1.Transfer 50 mL to a new 50 mL tube for use in preparing compound solutions and prewetting capture screens. Add fatty acid-free bovine serum albumin (BSA) to the remaining medium to achieve a final concentration of 0.2% (Table 2). Prewarm all media to 37 °C.

Table 1. Agilent Seahorse XF assay medium (no BSA).

Reagent	Product Number	[Final] (mM)	Volume (mL)
XF DMEM medium, pH 7.4	103575-100	-	100
XF 1.0 M glucose solution	103577-100	2.5	0.25
XF 200 mM glutamine solution	103578-100	2	1.0
XF 100 mM pyruvate solution	103579-100	1	1.0

Table 2. Preparation of 0.2% BSA assay medium.

Reagent	Final %	Stock Solution %	Volume (mL)
Seahorse XF assay medium (Table 1)	-	-	50
100 mg/mL BSA (fatty acid free)	0.2	10	1.0

Set up Seahorse XF Flex 3D capture microplate-S for islets

Prewet capture rings in a 60 mm petri dish containing XF assay medium without BSA. Use forceps to push the capture rings in medium with mesh side down and remove bubbles, if needed. Add 525 µL of 0.2% BSA assay medium to each well of XF 3D capture microplate-S and return to non-CO_2 incubator until ready to begin loading islets to the plate.

Add 6 mL 0.2% BSA assay medium to a 60 mm petri dish (sterile nontreated). Prewet pipet tips and other plastics in this medium to prevent islets from sticking to the surfaces of pipet tips and other plastics. Harvest sufficient islets for the experiment from the source plate by first swirling the source plate gently in the hood to allow for islets to gather in the center, then pick up islets with prewet pipette tips and transfer to the 60 mm dish containing 6 mL 0.2% BSA assay medium. Alternatively, islets may be harvested from culture dishes by removing the appropriate volume of islet culture based on the islet concentration and dispensing into a 15 mL tube. Allow islets to settle by gravity or centrifuge at 200 *x g* for two minutes. Remove the maintenance medium, resuspend islets in 0.2% BSA assay medium and place in a nontreated 60 mm petri dish.

Under a microscope, use a prewetted P10 pipette to collect islets from a petri dish in 4 to10 µL of assay medium, ensuring consistent size selection. To load into the capture plate, position the pipette tip through the medium to the bottom center of the well and slowly eject the islets into the central chamber. Gravity will help guide the islets down the tip; slow expulsion keeps them centered. **Do not load islets into wells A1 and D6**; they are background wells and should contain medium only.

Tip: Islets will begin to fall out of tip once ejection starts. Do not fully eject and push air into the well. Be certain to prewet tips and ensure there are no bubbles in the tip prior to picking islets.

Install prewet capture rings in all wells of plate, including background wells, by gently pushing the capture rings down into wells with the Agilent Seahorse XF 3D capture screen insert tool or forceps. Incubate the plate in a 37 °C non-CO_2 incubator for 30 to 45 minutes prior to assay. It is recommended to image the samples (brightfield) to evaluate placement.

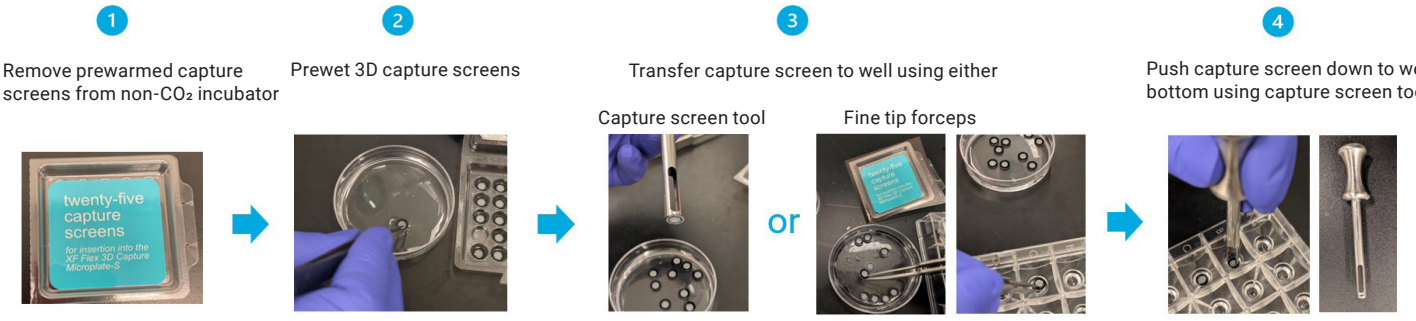

Figure 2. Installation of capture screens

Perform the Seahorse XF Mito Stress Test with islets

The preparation of sensor cartridges and the Agilent Seahorse XF Mito Stress Test compounds is detailed in the Agilent Seahorse XF 3D Mito Stress Test kit user guide. Table 3 and 4 below outline the preparation of stock and injection solutions. The Seahorse islet XF assay buffer with no BSA (Table 1) was used to prepare all compounds for injections. The glucose injection solution is prepared by diluting the Seahorse XF 1.0 M glucose solution, which is used in the first or the acute injection of the islet Mito Stress Test.

Ensure that the hydrobooster is removed before loading compounds to the injection ports on the cartridge.

Table 3. Preparation of stock solutions using the Agilent Seahorse XF 3D Mito Stress Test kit.

Reagent	Volume to Add (mL)	Stock Solution (µM)
Oligomycin A	2.7	270
FCCP	2.7	200
Rotenone/antimycin A	2.7	110

Table 4. Preparation of injection solutions for a starting well volume of 525 µL.

Injection Solution	Stock Solution (µL)	Assay Medium (µL)	Loading Port and Volume	[Final Well] (µM)
Glucose (8 x)	341 (1M Stock)	2659	Port A: 75 µL	16700 (+14200)
Oligo A (9 x)	500	2500	Port B: 75 µL	5
FCCP (10 x)	450	2550	Port C: 75 µL	3
*Rot/AA (11 x)	1500	1500	Port D: 75 µL	5

The XF Islet Mito Stress Test (Acute) assay template provided in the Agilent Seahorse Wave Controller software is optimized for islet samples with a default setting of a 3-minute mix, 0-minute wait, and 3-minute measure for each cycle. The number of cycles is set to four basal measurements, six after glucose injection, six after oligomycin A injection, three after FCCP injection and six after rotenone/antimycin A injection. Hoechst dye may be added in the last injection port with final concentration of 2 µM to allow for post assay fluorescence imaging of islets.

To run the XF Islet Mito Stress Test (Acute) assay select the XF Islet Mito Stress Test (Acute) assay template from the list of available templates in the XF Flex Controller software. Review the groups (cell types or conditions), plate layout map, and instrument protocol for your assay; modify if desired. On the review and run display, click Scan Assay Kit to display the assay kit information dialog. If available, use the external handheld barcode wand to scan the barcode on the kit box label (outside). This will automatically add the part number and lot number of the assay kit to this dialog. Otherwise, enter manually the kit information. In the SW ID field, manually type the software code on the kit box label. Click Apply. The software will embed this information into your data file. Click Start Run to start the assay. When prompted, remove the cartridge lid and place the loaded sensor cartridge with the utility plate on the thermal tray. Ensure correct plate orientation and that the cartridge lid and hydrobooster have been removed. Then, click I'm Ready. The calibration takes approximately 15 to 20 minutes. After completing calibration, click Open Tray to eject the utility plate and load the cell plate. Ensure that the lid is removed from the cell plate before loading. Click Load Cell Plate to run the assay.

Day of assay: Spheroid workflow

Prepare XF assay medium for spheroids

Prepare 100 mL of Agilent Seahorse XF DMEM assay medium according to Table 1, except for glucose. Instead of 2.5 mM, 10 mM glucose is used for spheroids. Warm the medium to 37 °C.

Set up Seahorse XF Flex 3D microplate for spheroids

Collect spheroids from the Eplasia culture plate by washing gently with 3 mL XF assay medium. Transfer to a 15 mL conical tube and allow them to settle by gravity (this may take 15 to 20 minutes). Slowly remove most of the medium, leaving about 500 µL to minimize disruption to the spheroids. Resuspend the spheroids in 5 mL of XF assay medium and transfer into a noncoated 60 mm petri dish for hand-picking directly into the XF 3D capture microplate as outlined above for islets.

Add 600 µL of XF assay medium to each well of the capture plate. Under a microscope, use a prewetted P10 pipette to collect desired number of spheroids from the petri dish in 4 to 10 µL of assay medium. Do not load spheroids into wells A1 and D6 as these are background wells and should contain medium only. Afterward, install prewet 3D capture rings to all wells following the instructions for islet assay setup.

Perform the Seahorse XF 3D Mito Stress Test with spheroids

In this study, the Agilent Seahorse XF Cell Mito Stress Test kit (part number 103015-100) was used as the amount of compounds provided in this kit is sufficient for HCT116-H2B-GFP spheroids ~ 150 µm. All compounds were prepared in assay medium according to Table 5 and 6. Ensure that the hydrobooster is removed before loading compounds to the injection ports on the cartridge.

Table 5. Preparation of stock solutions using the Agilent Seahorse XF Cell Mito Stress Test kit.

Compound	Volume to Add (µL)	Stock Solution (µM)
Oligomycin	630	100
FCCP	720	100
Rotenone/antimycin A	540	50

Table 6. Preparation of injection solutions for a starting well volume of 600 µL.

Injection Solution	Stock Solution (µL)	Assay Medium (µL)	Loading Port and Volume	[Final Well] (µM)
Oligo (9 x)	621	1679	Port B: 75 µL	3
FCCP (10 x)	230	2070	Port C: 75 µL	1
*Rot/AA (11 x)	506	1794	Port D: 75 µL	1

The 3D Mito Stress Test template provided in the Agilent Seahorse Wave Controller software was used and cycle numbers adjusted. This template is optimized for 3D samples with default setting of a 3-minute mix, 0-minute wait, and 3-minute measure for each cycle. The number of cycles was adjusted to three or four basal measurements, eight cyles after oligomycin injection, three after FCCP injection and six after rotenone/antimycin A injection. Hoechst dye may be added in the last injection port with final concentration of 2 µM to allow for post assay fluorescence imaging of spheroids.

Follow the instructions described in the islet workflow to run and complete the XF Mito Stress Test.

Day of assay: Imaging

Brightfield and fluorescent images at 4x magnification were captured using the Agilent BioTek Lionheart or Cytation imaging system before and after XF assay for evaluation of sample replacement and data normalization.

XF 3D Mito Stress Test data analysis

Analysis of XF 3D Mito Stress Test result files were performed using Seahorse Analytics, a web-based software platform. Using the XF 3D Mito Stress Test companion view in Seahorse Analytics, key assay parameters are automatically calculated, and presented in graphs.

Materials and equipment

Table 7. Materials and equipment used in workflow.

Material	Vendor	Part Number
Seahorse XF Flex 3D Capture FluxPak-S	Agilent Technolgies	103874-100
Seahorse XF 3D capture screen insert tool		103873-100
Seahorse XF 3D Mito Stress Test kit		103016-100
Seahorse XF Cell Mito Stress Test kit		103015-100
Seahorse XF DMEM pH7.4		103575-100
Seahorse XF 1.0 M glucose solution		103577-100
Seahorse XF 100 mM pyruvate solution		103578-100
Seahorse XF 200 mM glutamine solution		103579-100
Seahorse XF Flex analyzer		S7851A or S7851AN
Cytation imaging system		XXXXX
Lionheart imaging system		XXXXX

System performance evaluation

Islet assay

The typical results of an XF Mito Stress Test using 20 islets from wild type male Sprague Dawley rat are presented in Figure 3. This assay is the gold standard for assessing mitochondrial health, providing multiple parameters that are indicative of mitochondrial function, including basal respiration, ATP-linked respiration, maximal and reserve respiratory capacities, as well as nonmitochondrial respiration.

In the test, an acute injection of glucose was added to the assay protocol. As shown in Figure 3A, the islet samples exhibited robust OCR responses to all mitochondrial modulators as well as to high glucose concentration of 16.7 mM. The steady OCR signal from the control group (no compound injections) indicates that islets were healthy throughout the assay.

The overall ECAR signals were low, indicative of low glycolytic activity in the samples (Figure 3B). The increased ECAR signal post glucose injection and FCCP injection are likely due to mitochondrial CO_2 production and not glycolysis, as the ECAR and OCR trends followed a similar pattern and the inhibition of mitochondrial function by oligomycin or rotenone/antimycin A caused a reduction in ECAR. These observations are consistent with the fact that islets are a very oxidative sample type and do not have significant glycolytic activity.

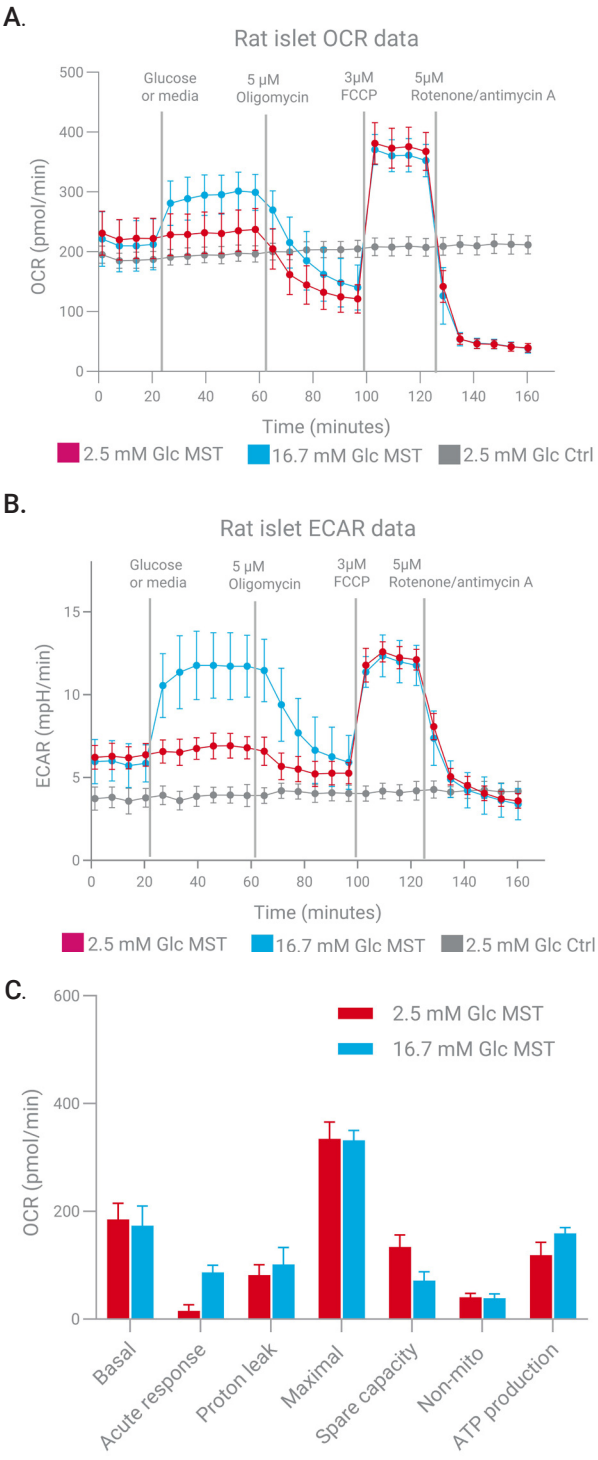

Figure 3. Typical results of Agilent Seahorse XF Mito Stress Test using the Agilent Seahorse XF 3D capture microplate-S on the Agilent Seahorse XF Flex analyzer, 20 islets per well. A. OCR kinetic graph. B. ECAR kinetic graph. C. Parameters reported by the XF Mito Stress Test.

Islets from a given pancreas vary in size and exhibit distinct metabolic characteristics. Their sensitivity to glucose differs, and mitochondrial respiration plays a critical role in glucose-stimulated insulin secretion. In the XF islet workflow, it is important to hand-pick islets of consistent size and number, and to place them directly in the center of the sample chamber of the XF 3D capture microplate-S. To ensure accurate and reliable results in downstream metabolic assays, it is important to avoid loading large islets that exhibit necrotic cores, as these may interfere with metabolic measurements.

During assay optimization, the number of islets (5, 10, 20 and 40) loaded per well was evaluated. A good correlation was observed between basal OCR and the number of islets from 5 to 40 islets per well (Figures 4A). Here, as little as 10 to 20 islets were sufficient to generate robust OCR signal (70 to 100 pmol/min). The number of islets should be optimized for the specific type and preparation used in the assay. Representative well images of stained islets post assay are shown in Figure 4B.

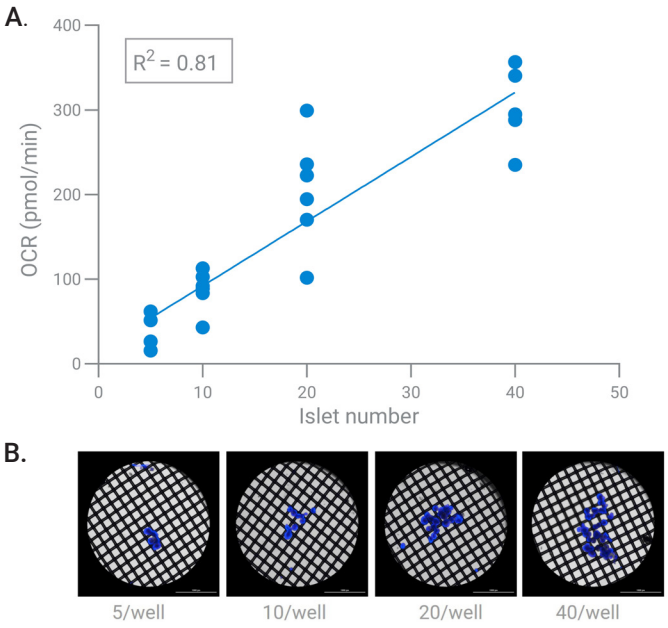

Figure 4. Correlation between OCR and islet number per well. B. Representative images of Hoechst-stained islets visualized under the capture screens of the Agilent Seahorse XF 3D capture microplate-S after Seahorse XF assay.

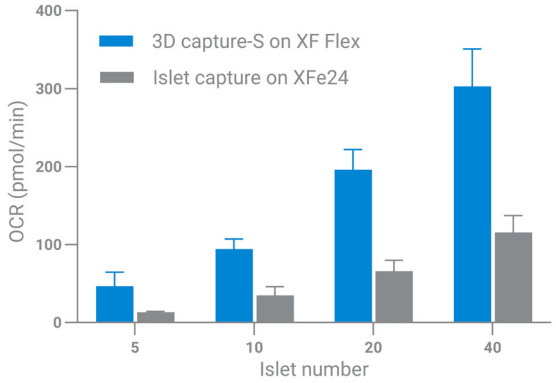

Figure 5. Comparison of basal OCR measurements with varying number of rat islets per well using the Agilent Seahorse 3D capture microplate-S on the Agilent Seahorse XF Flex analyzer and the Agilent Seahorse XF islet capture microplate on Agilent Seahorse XFe24 analyzer.

We also compared the performance of the assay using the XF 3D capture microplate-S on the XF Flex analyzer and the Agilent XF24 islet capture microplate on the Agilent Seahorse XFe24 analyzer. As shown in Figure 5, the basal OCR were much higher when using the XF 3D capture microplate-S on the XF Flex analyzer. In the XF Flex system, 10 to 20 islets generated basal OCR of ~ 100 to 200 pmol/min, while in XFe24 system, 40 islet are required to obtain basal OCR of 100 pmol/min, clearly demonstrating the increased sensitivity of the XF Flex system. The kinetic traces of the full XF Mito Stress Test performed on both systems are presented in Figure 6.

Along with the islet number, the dose of compounds should also be evaluated to determine the optimal concentrations. This is dependent on assay medium composition (with or without BSA or FBS), the islet source, size and number per well. Here, an example of determining the optimal FCCP concentration is presented where different FCCP doses were applied in two sequential injections after 16.7 mM glucose and 5 µM oligomycin A injections. This strategy provides a wider range of dose using fewer wells and islets. In Figure 7, we compare the maximum percent OCR after FCCP injection of 1, 2, 3, 4, 5 or 8 µM final FCCP

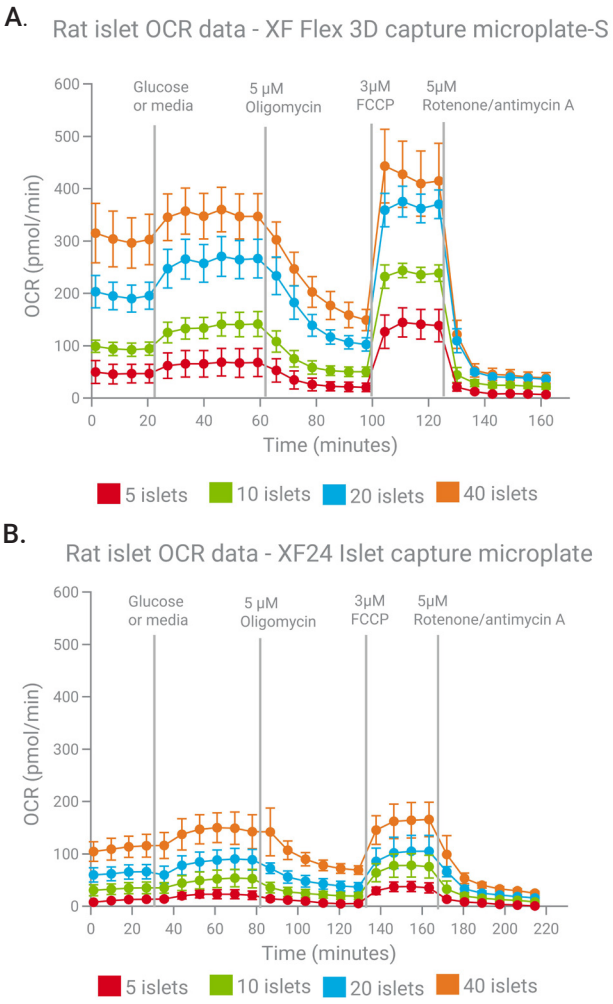

Figure 6. Performance comparison of the Agilent Seahorse 3D capture microplate-S on the Agilent Seahorse XF Flex analyzer (A) and the Agilent Seahorse XF islet capture microplate on Agilent Seahorse XFe24 analyzer (B), using 5, 10, 20 and 40 rat islets per well.

concentration. The data shows that 2 to 4 µM gave similar maximal OCR and 3 µM result appears to be more consistent (Figure 7B). Therefore, 3 µM is determined to be the optimal FCCP concentration. A concentration of 5 µM oligomycin and 5 µM rotenone/antimycin A were used in the islet assays.

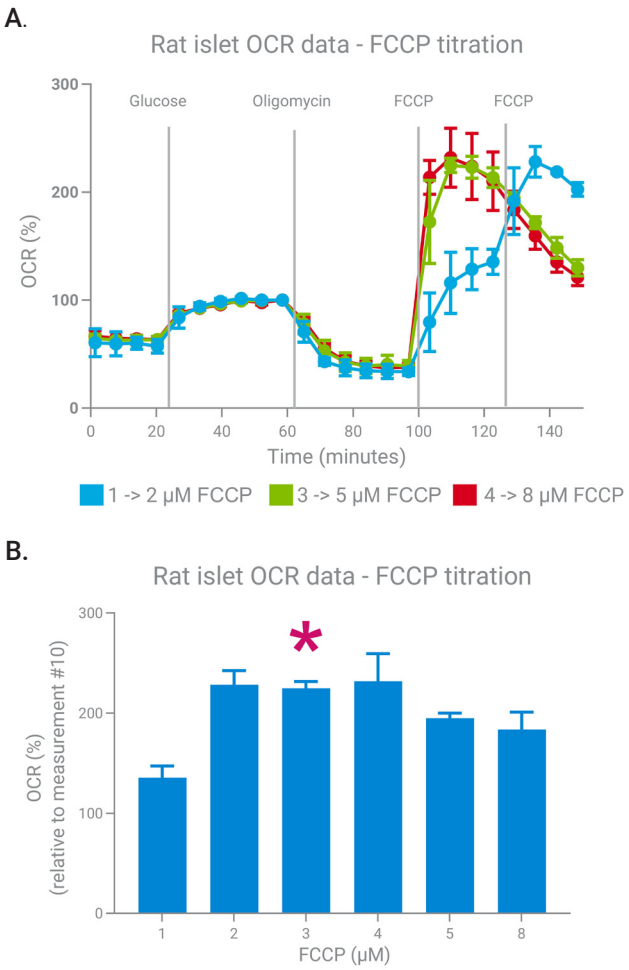

Figure 7. FCCP dose titration in rat islets using a sequential injection strategy. A. Kinetic graph of percent OCR baselined to measurement #10 (last measurement before oligomycin A injection). B. Bar graph of the maximal percent OCR after FCCP injections at different concentrations (the second measurement after each FCCP injection).

Spheroid assay

The typical results of an XF Mito Stress Test using seven-day old HCT116-H2B-GFP spheroids (average diameter 150 µm, 20 spheroids/well) are presented in Figure 8. Robust basal OCR of 200 pmol/min was observed. The spheroids also showed clear responses to all three modulators. As with the islets, it requires more measurement cycles for oligomycin to reach plateau effect. In the example shown here, eight cycles were allowed, but this can be adjusted based on spheroid number, size and type of spheroid used in the assay.

The HCT116-H2B-GFP spheroids are homogeneous samples, resulting in very consistent signals (7% CV for basal OCR from measurement 3). All key parameters indicating mitochondrial function were obtained using SHA software (Figure 8C).

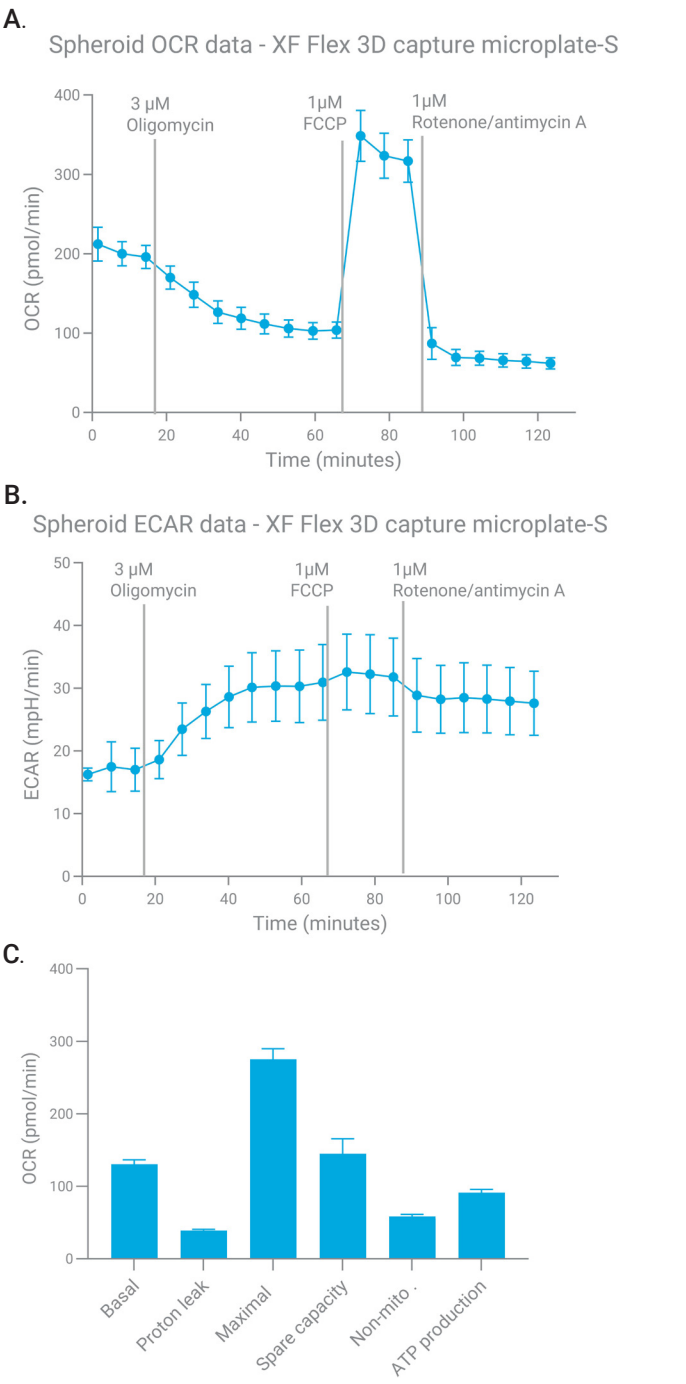

Figure 8. Typical Agilent Seahorse XF Mito Stress test results for HCT116-H2B-GFP spheroids (20) using the Agilent Seahorse XF 3D capture microplate-S on the Agilent Seahorse XF Flex analyzer. A. OCR kinetic graph. B. ECAR kinetic graph. C. Parameters reported by the XF Mito Stress Test.

The overall ECAR is low, but the ECAR kinetic trace does not mirror the OCR kinetic trace. When ATP synthase is shut down with oligomycin, a compensatory increase in ECAR is observed. This elevated ECAR is maintained after the injection of rotenone/antimycin A, indicating the increased ECAR activity is not mitochondrial respiration related, rather, likely an up-regulation of glycolysis compensating for the lost ability to produce ATP from mitochondrial respiration.

During assay optimization, the number of HCT116-H2B-GFP spheroids (10, 20, 40, and 60 per well) was evaluated using the XF 3D capture microplatelate-S. A linear relationship was observed from 10 to 60 spheroids (Figures 9) with a R^2 of 0.95. Such strong correlation and low variability is due to the homogeneous nature of the spheroids. Representative well images of stained spheroids post assay are shown in Figure 9B.

The XF 3D capture microplate-S features a 250 µm-deep sample chamber at the bottom of each well, along with a capture screen with a pore size of 170 µm. For this study, we used spheroids of approximately 150 µm diameter. The spheroid size range suitable for use with this microplate is between 150 to 300 µm.

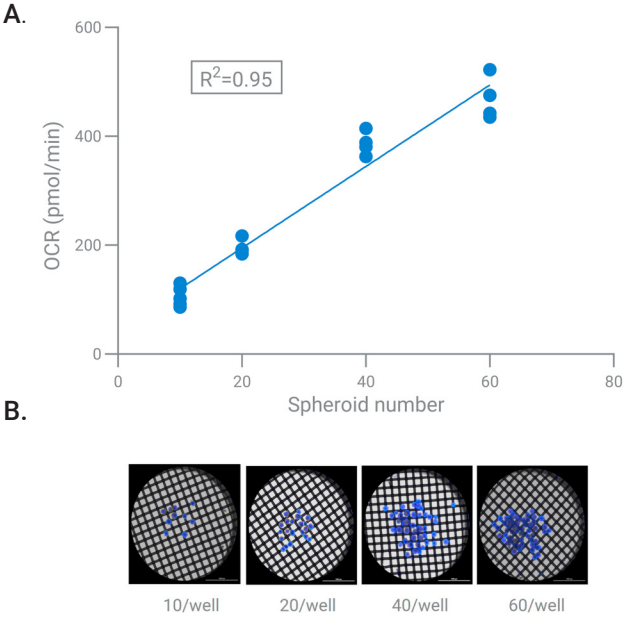

Figure 9. Basal OCR measurements with varying number of HCT116-H2B-GFP spheroids. A. Correlation between OCR and spheroid number per well. B. Image of spheroids stained with Hoechst dye, visualized under the capture screens after the Agilent Seahorse XF assay.

We also compared the assay performance using the XF 3D capture microplate-S on the XF Flex analyzer and the XF24 islet capture microplate on the XFe24 analyzer. As shown in Figure 10, the basal OCR for spheroids were approximately two-fold higher when using the XF 3D capture microplate-S on the XF Flex analyzer, further demonstrating the increased sensitivity of the XF Flex system.

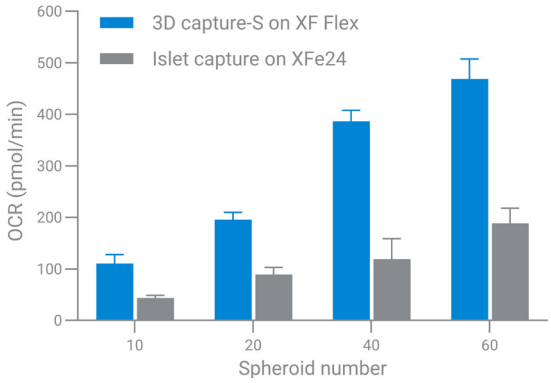

Figure 10. Comparison of basal OCR with varying number of HCT116-H2B-GFP spheroids using the Agilent Seahorse 3D Capture Plate-S on the Agilent Seahorse XF Flex analyzer and the Agilent Seahorse XF24 islet capture plate on the Agilent Seahorse XFe24 analyzer.

The optimal dose of compounds varies depending on the assay medium composition (with or without BSA or FBS), the spheroid cell type, preparation, size and number of spheroids per well. To determine the optimal FCCP concentration for HCT116-H2B-GFP spheroids, FCCP doses ranging from 0.25 to 2 µM were evaluated. Figure 11 shows that FCCP concentrations from 0.5 to 1.5 µM using 20 spheroids per well give similar and highest maximal OCR. Data using 1 µM is more consistent and represents the optimal concentration.

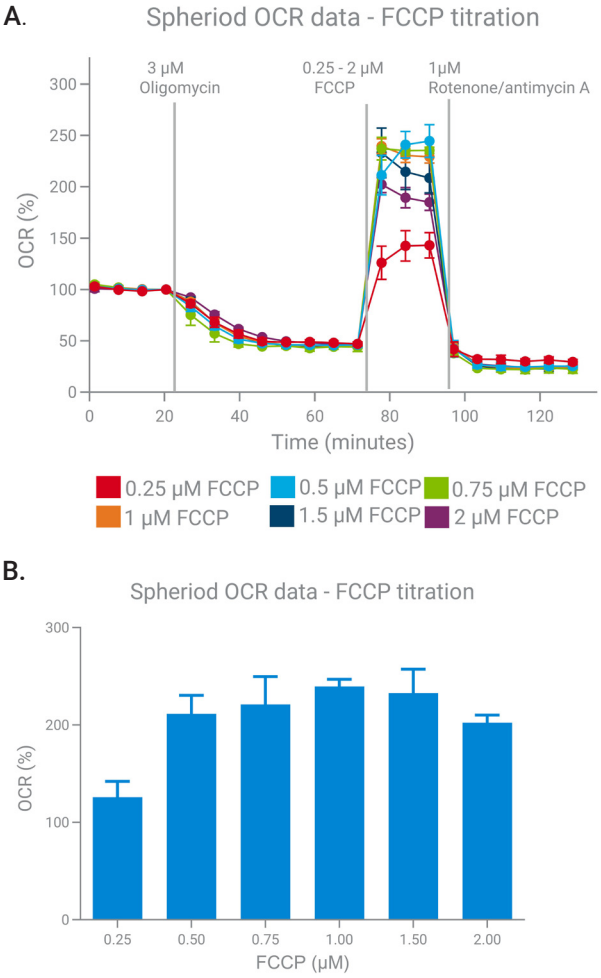

Figure 11. FCCP dose titration using HCT116-H2B-GFP spheroids. A. Kinetic graph of percent OCR baselined to measurement #4. B. Bar graph of the maximal percent OCR after FCCP injection (the first measurement after FCCP injection). Data from one representative experiment.

Data analysis and normalization considerations

Analysis of Seahorse XF metabolic assay data can be performed in Agilent Seahorse Analytics, a cloud-based software application, using the 3D Mito Stress Test kit. This companion view is available by entering the SW code on the product label of the XF Cell Mito Stress Test kit or the XF 3D Mito Stress Test kit. The 3D Mito Stress Test kit companion view offers widgets specifically designed for assays with 3D models, providing the options of data analysis without oligomycin as the first injection in standard Mito Stress Test and with oligomycin as the first or second injection in the case of an acute injection.

Normalization of Seahorse XF assay data is important in interpretation of assay results. It can minimize well-to-well variation caused by differences in sample size or numbers. The most appropriate method for normalization depends on the specific 3D sample used in the assay, the study objectives and assay conditions such as the medium composition. There are several approaches that can be considered to normalize data generated with 3D samples, such as imaging-based methods to obtain sample number/size, area, and fluorescence intensity from stained objects. In addition, percent of signal change relative to basal measurements or total protein or DNA content can be considered.

Imaging-based methods

Brightfield images can be acquired either before or after the assay to confirm sample positioning within the sample chamber. To enable fluorescent imaging of islets or spheroids, Hoechst dye may be included in the final injection of the rotenone/antimycin A solution. This approach allows for fluorescence imaging without interference from the screen, which can be present in brightfield images. Using Gen5 software, quantitative metrics such as total object area (ObjectSumArea) or total object intensity (ObjectSumInt) can be calculated for each well (Figure 12).

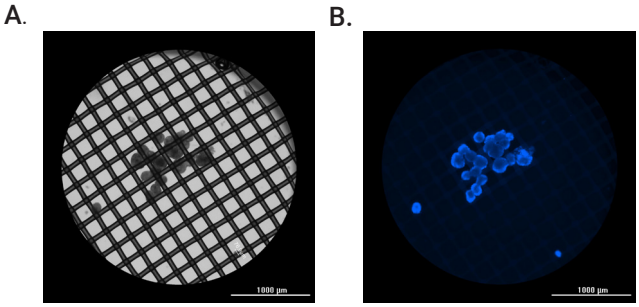

Figure 12. Islets in an Agilent Seahorse XF 3D capture microplate-S well before and after assay. A. Brightfield image prior to assay. B. Fluorescent image post assay with Hoechst 33342 stain.

As shown in Figure 13, a stronger linear correlation between oxygen consumption rate (OCR) and either Object Sum Area or Object Sum Intensity (fluorescence) was observed when islets were stained with Hoechst 33342 nuclear dye. This dataset corresponds to the data presented in Figure 4A, where OCR was plotted against islet number. Here, the coefficient of determination (R^2) improved modestly from 0.81 to a range of 0.88 to 0.91.

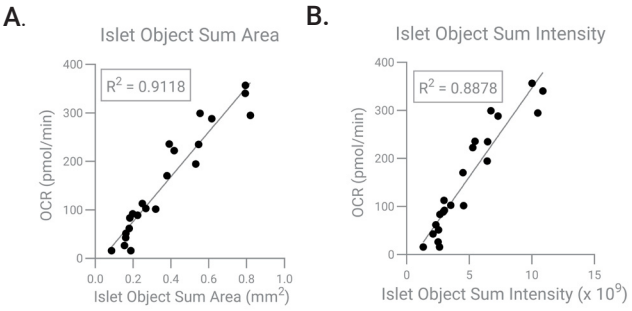

Figure 13. Linear correlation between OCR and islet Object Sum Area (A) or between OCR and islet Object Sum Intensity (B).

Using the same dataset, normalized OCR values are presented in Figure 14. Prior to normalization, basal OCR values ranged from 46 to 350 pmol/min across wells containing 5 to 40 islets. After normalization, the basal normalized OCR values for wells with 10, 20, and 40 islets varied by less than 15%, with a coefficient of variation (%CV) below 20% within each group. In contrast, wells containing only five islets exhibited high variability, indicating that this sample size is insufficient to generate robust assay data.

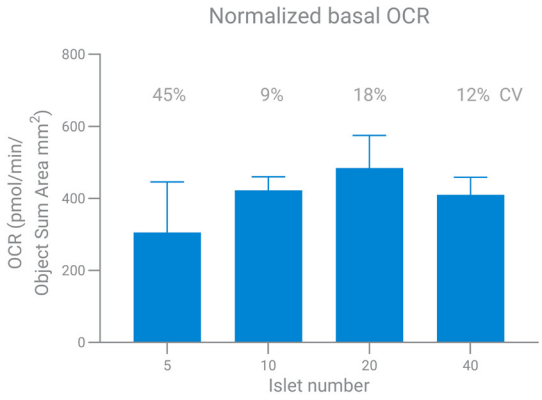

Figure 14. Basal OCR normalized to Object Sum Area (using the unnormalized raw data from Figure 4A).

In Figure 15, kinetic traces of the islet Mito Stress Test before normalization showed relatively large well-to-well variability (20 to 36% CV). When normalized to either total area (Figure 15B) or total intensity (Figure 15C), the data variability is improved. Normalizing the data to Object Sum Area reduced well-to-well variation to 12 to 14% CV, whereas normalization to Object Sum Intensity resulted in a CV range of 5 to 19%.

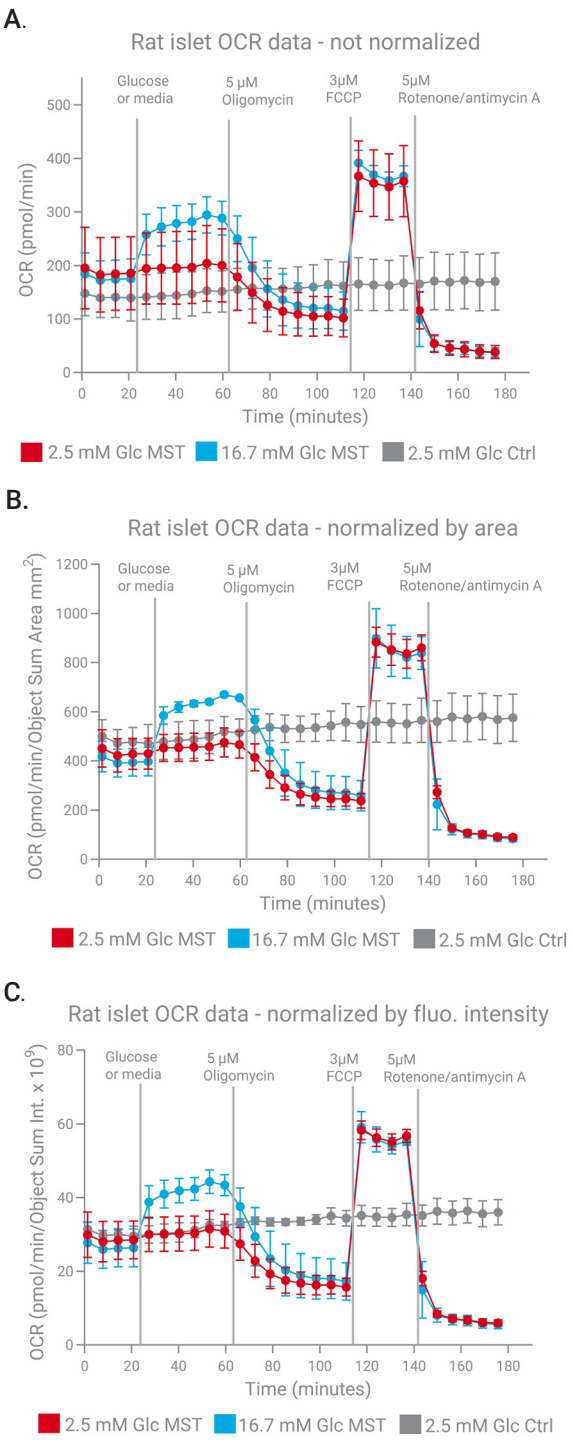

Figure 15. Agilent Seahorse XF Mito Stress Test with an acute glucose injection using the Agilent Seahorse 3D capture microplate-S on the Agilent Seahorse XF Flex analyzer, 20 rat islets per well. A. OCR kinetic graph before normalization. B. OCR kinetic graph normalized to object sum area. C. OCR kinetic graph normalized to object sum intensity.

Normalizing to basal signal

Baselining is another method that can be used to normalize Seahorse XF assay data. This is accomplished by converting the signals to a percentage relative to a basal measurement, usually the basal measurement right before the first injection. This method is applicable when a comparison of responses to an acute treatment or modulator is needed. It is especially useful for heterogenous samples. In Figure 16, islet OCR data from Figure 15A is baselined to measurement 4, the last measurement before glucose (or medium) injection, to assess the glucose response as percent of basal signal. Improvements in data consistency are observed for responses to glucose, oligomycin as well as rotenone/antimycin A.

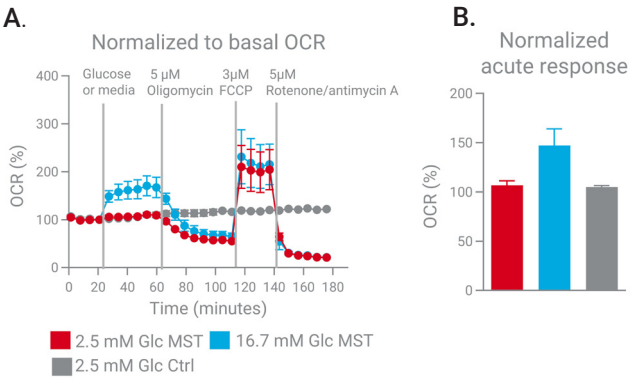

Figure 16. Agilent Seahorse XF Mito Stress Test with an acute glucose injection using rat islets (20 islets/well). A. OCR kinetic traces normalized to basal OCR (measurement 4). B. Bar chart of acute response to glucose injection normalized to basal OCR.

Other normalization options

There are additional methods that may be used, although they were not evaluated in this study. These include processing the islets or spheroids post assay for normalization by, for instance, lysing and quantifying protein or DNA content. However, these approaches may introduce variability and require additional processing time.

Islets primarily produce and secrete insulin, and many treatments can affect insulin RNA/protein. Therefore, using total RNA or protein as normalization metrics in islet assays is not recommended, because they could be affected by changes in insulin.

Conclusion

The Agilent Seahorse XF Flex analyzer along with the purpose-designed Agilent Seahorse 3D capture microplate-S dramatically improve sensitivity and efficiency, enabling robust metabolic profiling with as few as 10 islets per well. Unlike the older legacy Agilent Seahorse XFe24 analyzer and the Agilent Seahorse XF24 islet capture microplate which requires 40 to70 islets per well, the new XF Flex system is ideal for scarce or precious islet preparations, providing opportunity for more conditions and replicates per experiment. Improved sensitivity can lead to improved detection of subtle metabolic differences even in small islet groups. In addition, the example data using HCT116-H2B-GFP cancer spheroids demonstrates that this system is applicable to other types of small 3D materials.

Whether you are studying islet physiology, screening compounds, or evaluating donor islet viability, the XF Flex system empowers you to do more with less without compromising data quality.

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Products used in this application

Agilent products

Agilent Seahorse XF Flex analyzer

Agilent Seahorse XF Flex 3D capture FluxPak-S

Agilent Seahorse XF Flex 3D capture screen insert tool

Agilent Seahorse XF Flex 3D Mito Stress Test kit

Agilent Seahorse XF Flex Cell Mito Stress Test kit

Agilent Seahorse XF DMEM assay medium pack

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