Training Guide

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Cell Characterization: XFp Analyzer and the Cell Energy Phenotype Test

Basic Procedure

To effectively examine metabolic and bioenergetic function using the Agilent Seahorse XFp Extracellular Flux Analyzer, it is essential to first characterize a specific cell type with respect to its metabolic activity under basal and maximal respiration (OCR) and extracellular acidification (ECAR). The Seahorse XFp Cell Energy Phenotype Test Kit can be used to characterize the cell line/type of interest in 1-2 short assays.

There are two parameters which must be empirically determined to properly characterize cellular metabolic function: (1) the cell seeding density and (2) the concentration of FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), which is required to stimulate maximal oxygen consumption. Completion of these experiments provides an initial assessment of both the basal and maximal respiration rates of the cells, and verifies whether the chosen conditions provide rates within the dynamic range of the instrument for both OCR and ECAR values.

Optimal cell seeding number varies by cell type, but is typically between 5 x 10^3 and 4 x 10^4 cells per well. Generally, densities resulting in 50-90% confluency generate metabolic rates in the desirable/dynamic range of the instrument.

Please consult the following resources to provide an initial starting point for cell density values specific to your needs:

1. Cell Reference and/or XF publication data base: a searchable data base by cell type - http://www.agilent.com/cell-reference-database/ and http://www.agilent.com/publications-database/.
2. Assay Guides and Template Library: pre-made XF assay templates for many cell types with cell density and FCCP concentration values - http://www.agilent.com/en-us/support/cell-analysis-(seahorse)/seahorse-assay-guides-templates.

While suggested values may be found in the resources above, it is encouraged to still perform both cell density and FCCP titration analyses to ensure optimal cellular function under the assay conditions used.

Method

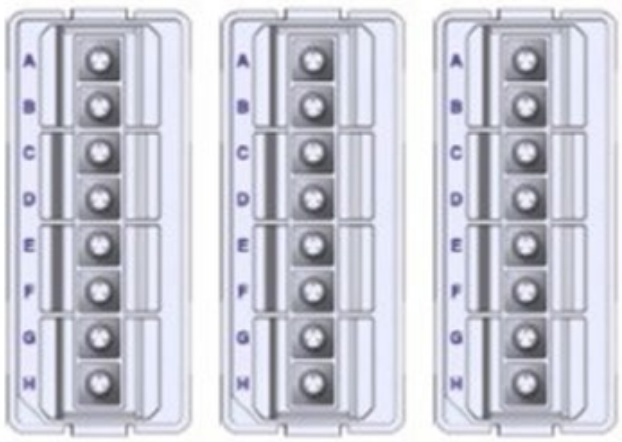
This method is for testing 1-3 different cell densities and five different FCCP concentrations using 1-2 XFp cell culture plates and sensor cartridges and the XFp Cell Energy Phenotype Test Kit with an XFp instrument.

There are two workflow options: (1) For cells that are NOT limited in number, multiple XFp cell culture miniplates can be seeded at different densities to reduce the time between experiments and complete the characterization workflow more quickly (Accelerated Workflow). (2) For cells limited in number, additional cells are prepared after the results of the first experiment are determined (Standard Workflow).

Step	Experiment	Rationale	Accelerated Workflow	Standard Workflow
1	Seed cells at single or different densities and visually assess degree of cell confluence; choose a miniplate for the next step.	To generate metabolic rates within the dynamic range of the instrument, cells should be 50-90% confluent. Visual assessment is a good first approximation of optimal cell density and will be verified in each assay.	Seed 3 miniplates at 3 different densities; hydrate 2 XFp cartridges.	Seed 1 miniplate at a single cell density; hydrate 1 XFp cartridge.
2	Perform a titration of FCCP in the presence of 1 µM oligomycin	Cell types vary in their response to the mitochondrial uncoupler FCCP, and at higher doses a reduced response can be observed. Therefore, the FCCP dose that generates the maximal OCR must be determined empirically.	Choose 1 plate from above based on visual inspection and perform a 5-point FCCP titration to generate dose response data.	Use the plate from above and perform a 5-point FCCP titration to generate dose response data.
3	Analyze Data: Verify chosen cell density and determine optimal FCCP concentration.	To establish and/or verify optimal cell seeding density based on basal and maximal OCR values. To establish and/or verify optimal FCCP concentration when using the established optimal cell density.	Examine the basal and maximal OCR rates; basal rate for cell density and max rate for FCCP concentration. If non-optimal, repeat assay using a plate seeded at a higher or lower density.	Examine the basal and maximal OCR rates; basal rate for cell density and max rate for FCCP concentration. If non-optimal, seed a plate a higher or lower density and perform assay the following day.

Day before Assay(s)

1. Choose 1-3 cell densities to test, based on standard or accelerated workflow described above. Either cover the range found in the references above, or seed the recommended cells/well value (1X) plus 0.5X cells/well and 2X cells/well cells per/well (e.g. $5x10^3$, $1x10^4$ and $2x10^4$ cells/well/plate).



Schematic of cell seeding strategy prior to the first XFp assay. Three miniplates at relative cell densities of 0.5x, 1x, and 2x are prepared and assessed visually. One plate is chosen to proceed to the first XF assay.

If performing the Accelerated Workflow, plate 1 miniplate of each cell density. XFp Miniplates have a well surface area of 40% of a standard 96-well plate, so scale accordingly. Note the time between cell seeding and performance of the assay.

For each cell density to be tested, seed as directed for either adherent or suspension cells.

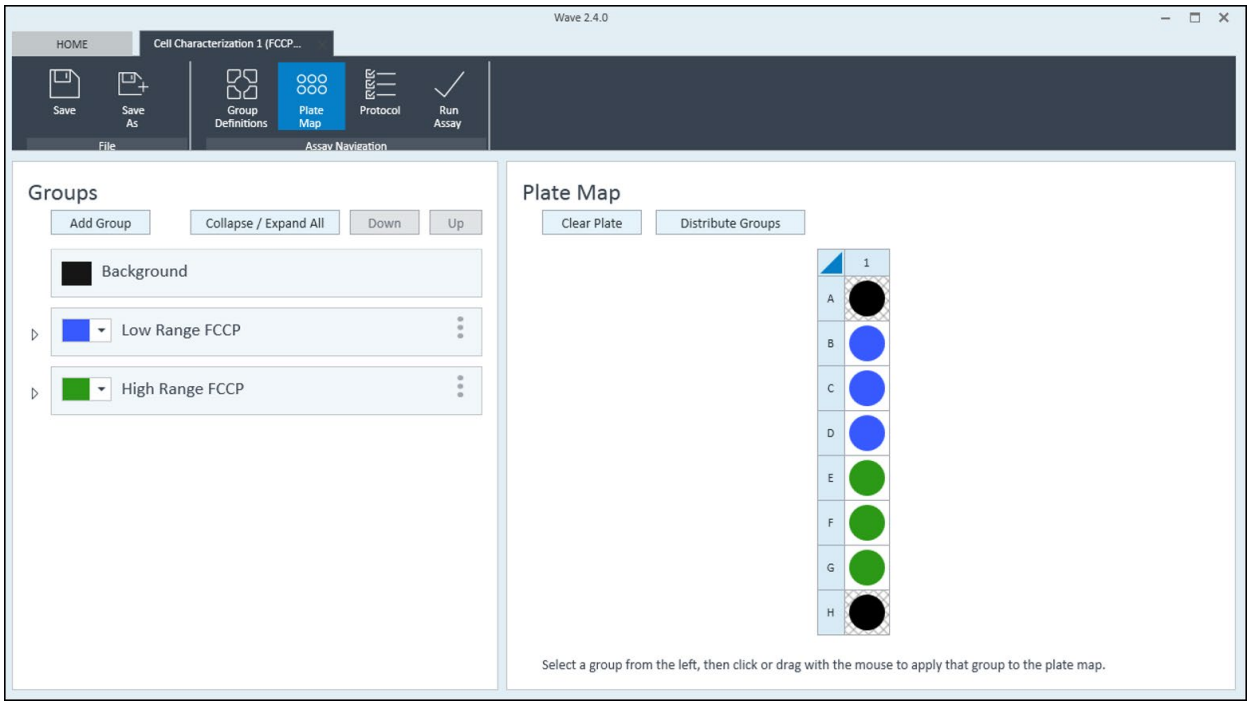
2. Adherent cell seeding procedure1: (to be performed day(s) prior to running an XFp assay) http://www.agilent.com/cs/library/usermanuals/public/Seeding%20Adherent%20Cells%20in%20XFp%20Cell%20Culture%20Miniplates.pdf

   Suspension cell seeding procedure: (to be performed just prior to running an XFp assay) http://www.agilent.com/cs/library/technicaloverviews/public/5991-7153EN.pdf.

3. Hydrate either 1 or 2 XFp cartridges the day prior to the XFp assay for the standard or accelerated workflow, respectively: http://www.agilent.com/cs/library/usermanuals/public/Hydrating%20an%20XFp%20Sensor%20Cartridge.pdf

Day of Assay(s)

1. Prepare XF Cell Energy Phenotype Assay Medium and warm to 37°C. Adjust pH to 7.4 ± 0.1 at 37°C. See: http://www.agilent.com/cs/library/usermanuals/public/Media%20Prep%20XFp.pdf .
2. Retrieve the cell culture plate(s) from the CO_2 incubator.
3. View the cells under the microscope to:
   a. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
   b. Ensure cells are adhered, with a consistent monolayer.
   c. Make sure there are no cells in the background correction wells
4. If using the accelerated workflow, choose the seeding density that produces 50-90% confluence with consistent monolayer. Wells seeded at the same density should appear consistent across the XFp cell culture miniplate.
5. Wash the cells with Seahorse XF Cell Energy Phenotype Assay Medium. Final well volume is 180 µL. http://www.agilent.com/cs/library/usermanuals/public/Washing%20Cells%20XFp.pdf .
6. View the cells under the microscope to ensure that cells were not disturbed or washed away.
7. Place the plate in a 37°C incubator **without CO_2** for one hour prior to the assay.
8. Design an assay template in the Wave software or XFp Analyzer by opening the *Cell Characterization 1 (FCCP Titration)* template.



1 Culture time depends on the cell type and the biological model: adherent vs. suspension, primary vs. transformed, and degree of differentiation required. Consult the literature for details about cell types and models of interest.

FCCP titration assay design

It is recommended to perform a 5-point titration curve to identify the FCCP concentration that yields maximal oxygen consumption rate (OCR). To do this, serial additions of FCCP are made using the injection ports in the XFp sensor cartridge. The benefit of this approach is that multiple data points for each dose are measured allowing a broad concentration curve to be generated in a single assay.

For this strategy, the XFp cartridge and cell culture plate are divided into 2 groups: a low FCCP concentration range (0.125, 0.25 and 0.50 µM, final) and a high FCCP concentration range (0.50, 1.0 and 2.0 µM, final), see figure below. Each group is first treated with 1 µM oligomycin, then three serial injections of FCCP at the different concentrations are performed. The resulting data set characterizes the cells' response to five different doses of FCCP

1. Prepare the XF Cell Energy Phenotype Test Injection Solutions as described below:

Resuspension volumes for the XFp Cell Energy Phenotype Test Kit				
XF Cell Energy Phenotype Test Component	**Volume of XF assay media (µl)**	**Resulting Stock Concentration (µM)**	**Working (Port) Concentration**	**Final (Well) Concentration (µM)**
Oligomycin	252	50	10	1.0
FCCP	288	50	1.25 - 10	0.125 - 2.0

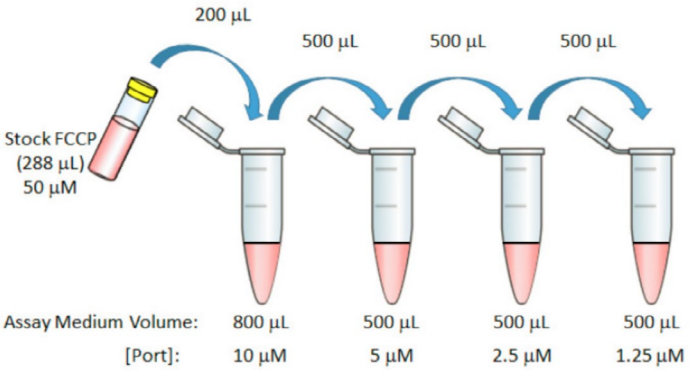
2. Using the 50 µM oligomycin stock, prepare the working concentration (10 µM) of oligomycin by mixing 60 µL of oligomycin stock with 240 µL of assay medium.

Dilution volumes for Oligomycin - Cell Characterization with XFp				
Ports Used for Oligo		**Volume of Assay Media (µl)**	**Volume of Stock Oligo (µl)**	**10X Final Oligo (Port) Concentration (µM)**
Low Range	A	240	60	10
High Range	A			

3. Prepare four separate dilutions of FCCP in assay medium, as detailed in the table below.

Dilution volumes for FCCP - Cell Characterization with XFp				
Ports Used for FCCP		Volume of Assay Media (µl)	Volume of Stock FCCP (µl)	10X Final FCCP (Port) Concentration (µM)
Low Range	B, C	487.5	12.5	1.25
	D	475	25	2.5
High Range	B, C	450	50	5
	D	400	100	10

Alternatively, serial dilutions of FCCP may be prepared by adding 800 µL assay medium to the first tube, and 500 µL assay medium to the other three tubes (see figure below).



Add 200 µL of FCCP stock (50 µM) into the first tube (containing 800 µL assay medium). Mix well, then serially dilute three times using 500 µL across the remaining tubes. This will yield FCCP injection port concentrations of 10 µM, 5 µM, 2.5 µM, and 1.25 µM.

4. Remove a hydrated XFp cartridge from the non-CO_2 incubator. Load each port of the cartridge as outlined in the table below and in the basic procedures document: http://www.agilent.com/cs/library/usermanuals/public/Loading%20Cartridge%20XFp.pdf

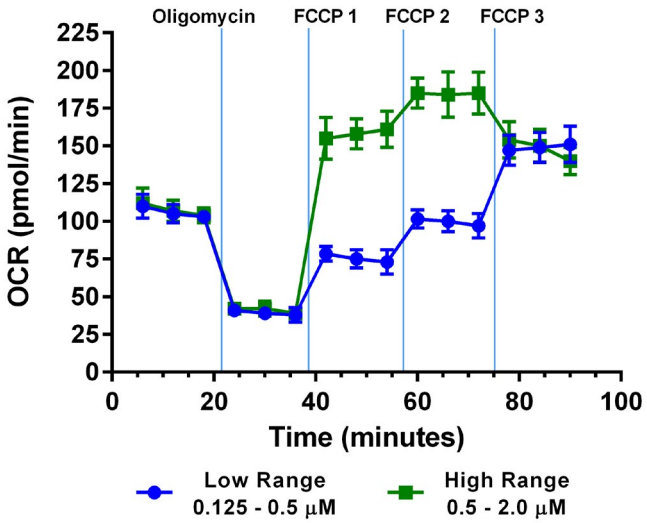
Port Designation	Volume per Port	Port Concentration Wells A-D (Low Range)	Port Concentration Wells E-H (High Range)	Final Well Concentration Wells A-D (Low Range)	Final Well Concentration Wells E-H (High Range)
A (Oligomycin)	20 µL	10 µM	10 µM	1 µM	1 µM
B (FCCP)	22 µL	1.25 µM	5 µM	0.125 µM	0.5 µM
C (FCCP)	28 µL	1.25 µM	5 µM	0.25 µM	1.0 µM
D (FCCP)	30 µL	2.5 µM	10 µM	0.5 µM	2.0 µM

NOTE: Fill the ports of all wells, including those corresponding to the background wells, to ensure successful injections

5. Once all ports are filled, transfer the cartridge and utility plate to the XFp instrument and begin cartridge calibration using the assay template created above.
6. Once cartridge calibration is complete, follow the prompts in the Wave software to exchange the utility plate for the cell culture plate and initiate the XFp assay.
7. When the assay is complete, eject the cartridge/cell plate assembly and set aside for later analysis. Save the Wave Results file to a shared folder on your local network or to a USB drive, and then open on a PC or laptop using the Wave Desktop software.

Verification of Cell Density and Determine Optimal Dose of FCCP

The next step is to examine the data and plan for the next experiment. Open the results file in Wave and examine the OCR data, which should look similar to the data shown below:

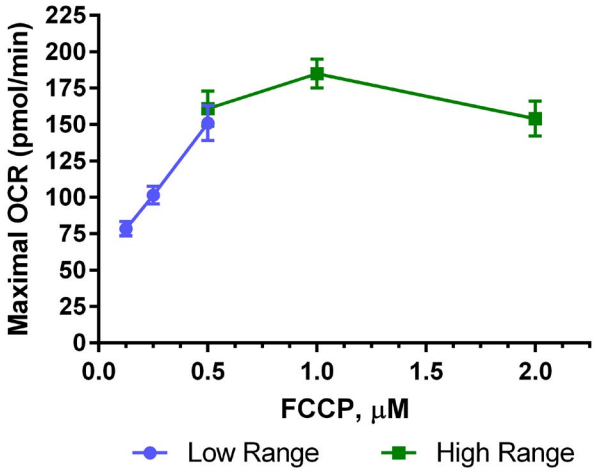


Sample data from an FCCP titration performed as described above in a Seahorse XFp Analyzer. The blue line represents wells in the Low Range group and the green line shows the results for the High Range well group Note as the FCCP concentration increases, the OCR value also increases up to 1.0 µM FCCP. Upon addition of more FCCP (2.0 µM final), the OCR begins to decrease, indicating that 1.0 µM is an optimal concentration of FCCP for this cell type under these assay conditions.

The data generated will help determine: (1) whether the cell density is within the optimal range: basal OCR values between 20-150 pmol/min, and (2) what dose of FCCP should be used to elicit maximal respiration in these cells (i.e. the lowest FCCP concentration which yields the highest OCR).

The figure above shows OCR vs. Time and the effect of serial injections of FCCP on respiration rate (OCR). For the low range FCCP group (blue trace), the OCR increases with increasing concentrations of FCCP. However, at higher doses (green trace) the response reaches a plateau, then decreases slightly.

The figure below shows the maximal FCCP OCR data transformed into a dose response curve: OCR vs. [FCCP]:



In this instance, 1.0 uM would be chosen as the optimal FCCP concentration for this cell type under these assay conditions, as it is the FCCP dose that elicits the maximal response in respiration.

Further optimization of cell density and FCCP concentration

- In instances where the basal OCR values are *below* the recommended range of 20-150 pmol/min, then repeat the assay using an XFp cell culture plate seeded at a *higher* cell density.
- In instances where the basal OCR values are *below* the recommended range of 20-150 pmol/min, then repeat the assay using an XFp cell culture plate seeded at a *higher* cell density.
- Repeat data analysis as described above to determine if the basal OCR is now in the recommended range and check the optimal FCCP concentration with the lower/higher cell seeding density.
- See Training Module 4: Agilent Seahorse Software Overview: Wave and Report Generators for further instructions and guidance on data analysis and interpretation for choosing optimal cell seeding density and FCCP concentration.

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