Training Guide

Agilent
Trusted Answers

Cell Characterization: The XFe24/XF24 Analyzer and the Cell Energy Phenotype Test

To effectively examine metabolic and bioenergetic function using the Agilent Seahorse XFe24 or XF24 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 XF Cell Energy Phenotype Kit can be used to characterize the cell line/type of interest in two 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 1×10^4 and 8×10^4 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.

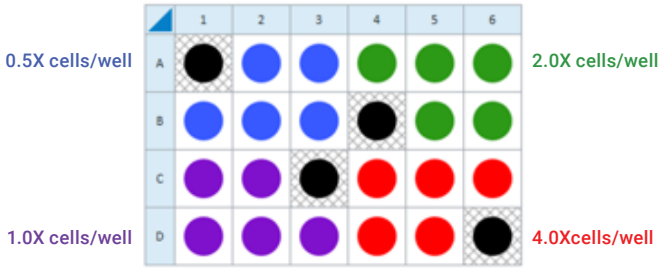
Method

This method is for testing four different cell densities and four different FCCP concentrations using two XF24 cell culture plates and two XFe24 or XF24 cartridges and the XF Cell Energy Phenotype Test Kit with an XFe24 or XF24 instrument.

Day before Assay 1: Seeding cells for Cell Density Titration

1. Choose four cell densities to test. Either cover the range found in the references above, or seed the recommended cells/well value (1X) plus 0.5X cells/well, 1X, 2X cells/well and 4X cells per/well (e.g. $1x10^4$, $2x10^4$, $4x10^4$, $8x10^4$ cells/well).

   This is a suggested XFe24/XF24 assay plate map for seeding four cell densities:



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

   Adherent cell seeding procedure[1]: (to be performed day(s) prior to running an XF assay)

   http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_BEFORE_CELL_SEEDING.pdf or

   http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_BEFORE_CELL_SEEDING.pdf

   Suspension cell seeding procedure: (to be performed just prior to running an XF assay) http://www.agilent.com/cs/library/technicaloverviews/public/5991-7154EN.pdf

3. Hydrate an XFe24 or XF24 cartridge the day prior to the XF assay: http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf

Day of Assay 1: Cell Density Titration

1. Prepare Seahorse XF Cell Energy Phenotype Test 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/XFe24_DAY_OF_MEDIA_PREP.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_MEDIA_PREP.pdf .

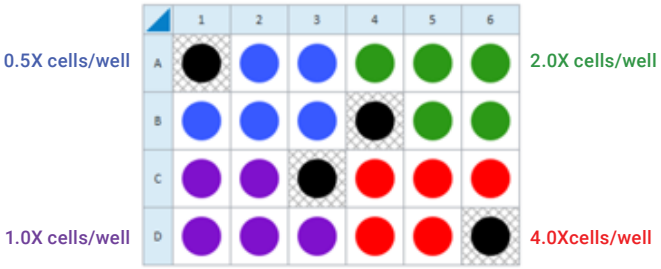
2. Retrieve the cell culture plate from the CO_2 incubator.
3. View the cells under the microscope to:
   a. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
   b. Ensure cells are adhered, with a consistent monolayer.
   c. Make sure there are no cells in the background correction wells
4. Wash cells with Seahorse XF Cell Energy Phenotype Test Assay Medium. Final well volume is 500 µL. http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_OF_WASHING_CELLS.pdf or

   http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_WASHING_CELLS_XF24.pdf

5. View the cells under the microscope to ensure that cells were not disturbed or washed away.
6. Place the plate in a 37°C incubator without CO_2 for one hour prior to the assay.

[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.

7. Design an assay template in the Wave software by opening the XF Cell Energy Phenotype Test Template. Below is a suggested plate map for testing the four different cell densities seeded:



8. Prepare the XF Cell Energy Phenotype Test Injection Solution as described below:

Resuspension volumes for the XF Cell Energy Phenotype Test Kit		
XF Cell Energy Phenotype Test Component	**Volume of XF assay media (µl)**	**Resulting Stock Concentration (µM)**
Oligomycin	630	100
FCCP	720	100

Dilution volumes for the Cell Energy Phenotype Test Kit - Cell Characterization with XFe24/XF24					
Final FCCP concentration in well	**Volume of Assay Media (µl)**	**Volume of Stock Oligomycin (µl)**	**Volume of Stock FCCP (µl)**	**10X Final Oligo (Port) Concentration (µM)**	**10X Final FCCP (Port) Concentration (µM)**
1.0	2400	300	300	10	10

9. Remove the hydrated cartridge from the non-CO_2 incubator. Load each A port of the XFe24 or XF24 cartridge as outlined below and described at: http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_OF_LOADING_CARTRIDGE.pdf or

   http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_LOADING_CARTRIDGE.pdf, respectively.

Final Concentration in well (µM)	Groups	Wells	Port/Volume (µl)
Oligo / FCCP 1.0 / 1.0	All	A1-D6	A/55

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

10. Once all required ports are filled, transfer the cartridge and utility plate to the XFe24/XF24 instrument and begin cartridge calibration using the assay template created in step 7 above.
11. 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 XF assay.
12. 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. See Training Module 4: Agilent Seahorse Software Overview: Wave and Report Generators for instructions and guidance on data analysis and interpretation for choosing the optimal cell density and FCCP concentration.

Day before Assay 2: Seeding cells for FCCP Titration

1. Choose the optimal cell seeding density based on the results of Assay 1 above.
2. For the cells to be tested, seed an XFe24 or XF24 cell culture plate at this optimal density as directed for either adherent or suspension cells.

   Adherent cell seeding procedure: (to be performed day(s) prior to running an XF assay)

   http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_BEFORE_CELL_SEEDING.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_BEFORE_CELL_SEEDING.pdf

   Suspension cell seeding procedure: (to be performed just prior to running an XF assay)

   http://www.agilent.com/cs/library/technicaloverviews/public/5991-7154EN.pdf
3. Hydrate an XFe24/XF24 cartridge the day prior to the XF assay: http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf

Day of Assay 2: FCCP titration

1. Prepare XF Cell Energy Phenotype Test 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/XFe24_DAY_OF_MEDIA_PREP.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_MEDIA_PREP.pdf

2. Retrieve the cell culture plate 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. Wash cells with XF Cell Energy Phenotype Test Assay Medium. Final well volume is 500 µL. http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_OF_WASHING_CELLS.pdf or

   http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_WASHING_CELLS_XF24.pdf
5. View the cells under the microscope to ensure that cells were not disturbed or washed away.
6. Place the plate in a 37°C incubator without CO_2 for one hour prior to the assay.
7. Design an assay template in the Wave software by opening the XF Cell Energy Phenotype Test Template. Below is a suggested plate map for testing four different concentrations of FCCP:
8. Prepare XF Cell Energy Phenotype Test Injection Solutions as described in the tables below:

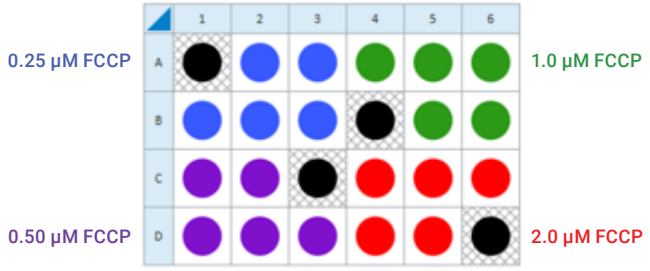
Resuspension volumes for the XF Cell Energy Phenotype Test Kit		
XF Cell Energy Phenotype Test Component	**Volume of XF assay media (µl)**	**Resulting Stock Concentration (µM)**
Oligomycin	630	100
FCCP	720	100

Dilution volumes for the Cell Energy Phenotype Test Kit - Cell Characterization with XFe24/XF24					
Final FCCP concentration in well	**Volume of Assay Media (µl)**	**Volume of Stock Oligomycin (µl)**	**Volume of Stock FCCP (µl)**	**10X Final Oligo (Port) Concentration (µM)**	**10X Final FCCP (Port) Concentration (µM)**
0.25	875	100	25	10	2.5
0.5	850	100	50	10	5.0
1.0	800	100	100	10	10
2.0	700	100	200	10	20

9. Remove the hydrated cartridge from the non-CO_2 incubator. Load each A port of the XFe24 or XF24 cartridge as outlined below and described at: http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_LOADING_CARTRIDGE.pdf or

   http://www.agilent.com/cs/library/usermanuals/public/XF24_DAY_OF_LOADING_CARTRIDGE.pdf, respectively.

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



Final Concentration in well (µM)	FCCP Group	Wells	Port / Volume (µl)
Oligo / FCCP 1.0 / 0.25 µM	0.25 µM	A1-A3, B1-B3	A / 55
Oligo / FCCP 1.0 / 0.50 µM	0.50 µM	A4-A6, B4-B6	A / 55
Oligo / FCCP 1.0 / 1.0 µM	1.0 µM	C1-C3, D1-D3	A / 55
Oligo / FCCP 1.0 / 2.0 µM	2.0 µM	C4-C6, D4-D6	A / 55

10. Once all required ports are filled, transfer the cartridge and utility plate to the XFe24/XF24 instrument and begin cartridge calibration using the assay template created in step 7 above.
11. 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 XF assay.
12. 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. See Agilent Seahorse XF Training Module 4: Wave Software Overview and Data Analysis for instructions and guidance on data analysis and interpretation for choosing optimal FCCP concentration.

www.agilent.com/chem/discoverxf

Printed in the USA, January 4, 2018
5991-8640EN

Agilent
Trusted Answers