Training Guide

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Cell Characterization: The XFe96/XF96 Analyzer and the XF Real Time ATP Rate Assay

To effectively examine metabolic and bioenergetic function using the Agilent Seahorse XFe96 or XF96 Extracellular Flux Analyzer, it is essential to first characterize a specific cell type with respect to its metabolic activity under basal respiration (OCR) and extracellular acidification (ECAR). The XF Real Time ATP Rate Assay can be used to characterize the cell line/type of interest in a single assay plate.

A key parameter which must be empirically determined to properly characterize cellular metabolic function is the cell seeding density. Completion of this experiment also provides an initial assessment of the basal respiration rate of the cells, and verifies whether the chosen conditions provide rates within the dynamic range of the instrument for both OCR and ECAR values. In addition, this assay will provide a powerful way to measure cell function by kinetic quantification of ATP production, including for mitoATP and glycoATP production rates, as well as the total ATP production rates.

Optimal cell seeding number varies by cell type, but is typically between 0.5 x 10^4 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 cell density titration analysis to ensure optimal cellular function under the assay conditions used.

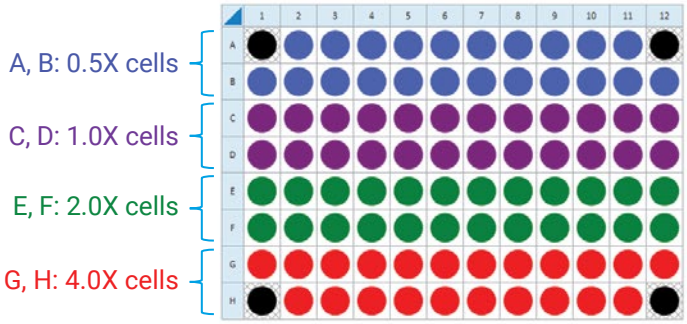
Method

This method is for testing four different cell densities using an XF96 cell culture plate, XFe96 cartridge and the XF Real Time ATP Rate Assay Kit with an XFe96 or XF96 instrument.

Day before Assay

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, 2X cells/well and 4X cells per/well (e.g. 0.5x10^4, 1x10^4, 2x10^4, 4x10^4 cells/well).

   This is a suggested XFe96/XF96 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[1].

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

   http://www.agilent.com/cs/library/usermanuals/public/XFe96_DAY_BEFORE_CELL_SEEDING.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF96_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/5991-7153EN.pdf.

3. Hydrate an XFe96 cartridge the day prior to the XF assay: http://www.agilent.com/cs/library/usermanuals/public/XFe96_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf.

[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.

Day of Assay

1. Prepare XF Real Time ATP Rate Assay Medium and warm to 37°C.

Agilent Reagent / Agilent Part Number	Final Concentration	Volume
Seahorse XF DMEM Medium, pH 7.4[a] / 103575-100 or Seahorse XF RPMI Medium, pH 7.4[a] / 103576-100	-	97.0 mL
Seahorse XF Glucose (1.0 M solution) / 103577-100	10 mM	1.0 mL
Seahorse XF Pyruvate (100 mM solution) / 103578-100	1 mM	1.0 mL
Seahorse XF L-Glutamine (200 mM solution) / 103579-100	2 mM	1.0 mL
[a]XF DMEM and RPMI Medium, pH 7.4 have a pre-adjusted pH value and do not require adjustment of pH upon addition of XF supplements. See method below for preparation.		

   See: http://www.agilent.com/cs/library/usermanuals/public/XFe96_DAY_OF_MEDIA_PREP.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF96_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 **one** time with XF Real Time ATP Rate Assay Medium. Final well volume is 180 µL. http://www.agilent.com/cs/library/usermanuals/public/XFe96_DAY_OF_WASHING_CELLS.pdf or http://www.agilent.com/cs/library/usermanuals/public/XF96_DAY_OF_WASHING_CELLS.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 Real-Time ATP Rate Assay Template. Above is a suggested plate map for testing four different cell densities.
8. Prepare the XF Real Time ATP Rate Assay Stock Compounds and Injection Solutions as described below:

Resuspension volumes for the XF Real Time ATP Rate Assay Kit		
Compound	Volume of XF Assay Media	Resulting Stock Concentration
Oligomycin	420 µl	150 µM
Rotenone + Antimycin A	540 µl	50 µM

Dilution volumes for XF Real Time ATP Rate Assay Kit - Cell Characterization				
Port & Compound	Stock Volume	XF Assay Media Volume	10X [Port]	[Final Well]
Port A Oligomycin	300 µl	2700 µl	15 µM	1.5 µM
Port B Rotenone + Antimycin A	300 µl	2700 µl	5 µM	0.5 µM

9. Remove the hydrated cartridge from the non-CO_2 incubator. Load each port A of the cartridge as outlined below and described at: http://www.agilent.com/cs/library/usermanuals/public/DAY%20OF%20LOADING%20CARTRIDGE%20XFe96-XF96.pdf

Injection Port volumes for XF Real Time ATP Rate Assay Kit - Cell Characterization			
Port & Compound	Volume	10X [Port]	[Final Well]
Port A Oligomycin	20 µl	15 µM	1.5 µM
Port B Rotenone + Antimycin A	22 µl	5 µM	0.5 µM

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 XFe96/XF96 instrument and begin cartridge calibration using the assay template created in step 7 above.
11. Once cartridge calibration is complete, wash the cells again with XF Real-Time ATP Rate Assay Media (final volume 180 µL), and inspect the cells under the microscope to ensure that cells were not disturbed or washed away. Then 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.

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