Training Guide

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Cell Characterization: The XF HS Mini Analyzer and the XF Real-Time ATP Rate Assay

Basic procedure

To effectively examine metabolic and bioenergetic function using the Agilent Seahorse XF HS Mini 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 Seahorse XFp Real-Time ATP Rate Assay can be use to characterize the cell line/type of interest in two short assays.

A key parameter which must be empirically determined to properly characterize cellular metabolic function is the cell seeding density. Completion of these experiments provides an initial assessment of the basal 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. 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 0.5×10^4 and 4×10^4 cells per well for adherent cells and between 5.0×10^4 and 3.0×10^5 cells per well for suspension cells. Generally, densities resulting in 50 to 90% confluency generate metabolic rates in the desirable/dynamic range of the instrument.

Experiment	Rationale	Accelerated Workflow	Standard Workflow
Seed cells at different densities and visually assess degree of cell confluence; choose an XFp miniplate for the next step.	To generate metabolic rates within the dynamic range of the instrument, cells should be 50 to 90% confluent. Visual assessment is a good first approximation of optimal cell density and will be verified in each assay.	Seed 1 to 2 XFp miniplates at 2 to 4 different densities; hydrate 1 to 2 XFp cartridges.	Seed 1 XFp miniplate at a single cell density; hydrate one XFp cartridge.

Please consult the XF Publication Database to provide an initial starting point for cell density values specific to your needs: http://www.agilent.com/publications-database/.

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

Method

This method is for testing 2 to 4 different cell densities using 1 to 2 XFp cell culture plates and sensor cartridges and the XFp Real-Time ATP Rate Assay Kit with an XF HS Mini Analyzer.

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).

Day before Assay(s)

1. Choose 2 to 4 cell densities to test, based on the 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, 2.0x, and 4.0x cells/well (Figure 1).

For each cell density to be tested, seed as directed for either adherent or suspension cells[1].

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

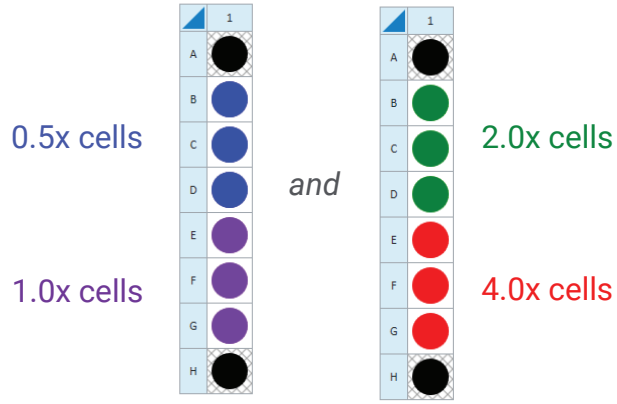

Figure 1. If performing the Accelerated Workflow, seed 3 wells of each cell density in 2 XFp Miniplates. XFp Miniplates have a well surface area of 40% of a standard 96-well plate, so scale accordingly. Note the time between cell seeding and performance of the assay.

2. Adherent cell seeding procedure: (to be performed day(s) prior to running an XF HS Mini assay using an XFp Miniplate) 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: https://www.agilent.com/cs/library/usermanuals/public/Hydrating_an_XFp_Sensor_Cartridge.pdf

Day of Assay(s)

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

Agilent Reagent / Agilent Part Number	Final Conc.	Volume
Seahorse XF DMEM Medium, pH 7.4[a]/103575-100 or Seahorse XF RPMI Medium, pH 7.4[a]/103576-100	–	9.70 mL
Seahorse XF Glucose (1.0 M solution)/103577-100	10 mM	100 µL
Seahorse XF Pyruvate (100 mM solution)/103578-100	1 mM	100 µL
Seahorse XF L-Glutamine (200 mM solution)/103579-100	2 mM	100 µL
[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 the method below for preparation.		

See: http://www.agilent.com/cs/library/usermanuals/public/Media%20Prep%20XFp.pdf .

2. If using suspension cells, follow the recommended suspension cells seeding procedure and skip to step 7. If using adherent cells, 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 that the 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 to 90% confluence with consistent monolayer. Wells seeded at the same density should appear consistent across the XFp cell culture miniplate.
5. Wash the 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/Washing%20Cells%20XFp.pdf
6. Place the plate in a 37 °C incubator **without CO_2** for one hour prior to the assay.
7. Design an assay template using the Seahorse XF Real-Time ATP rate assay template on the Seahorse XF HS Mini analyzer (Figure 2).
8. Prepare the XFp Real-Time ATP Rate Assay Stock Compounds and Injection Solutions as described below:

Resuspension volumes for the XFp Real Time ATP Rate Assay Kit		
Compound	**Volume of XF Assay Media**	**Resulting Stock Concentration**
Oligomycin	168 µL	75 µM
Rotenone + Antimycin A	216 µL	25 µM

Dilution volumes for XFp Real Time ATP Rate Assay Kit – Cell Characterization				
Port and Compound	**Stock Volume**	**XF Assay Media Volume**	**10X [Port]**	**[Final Well]**
Port A Oligomycin	60 µL	240 µL	15 µM	1.5 µM
Port B Rotenone + Antimycin A	60 µL	240 µL	5 µM	0.5 µM

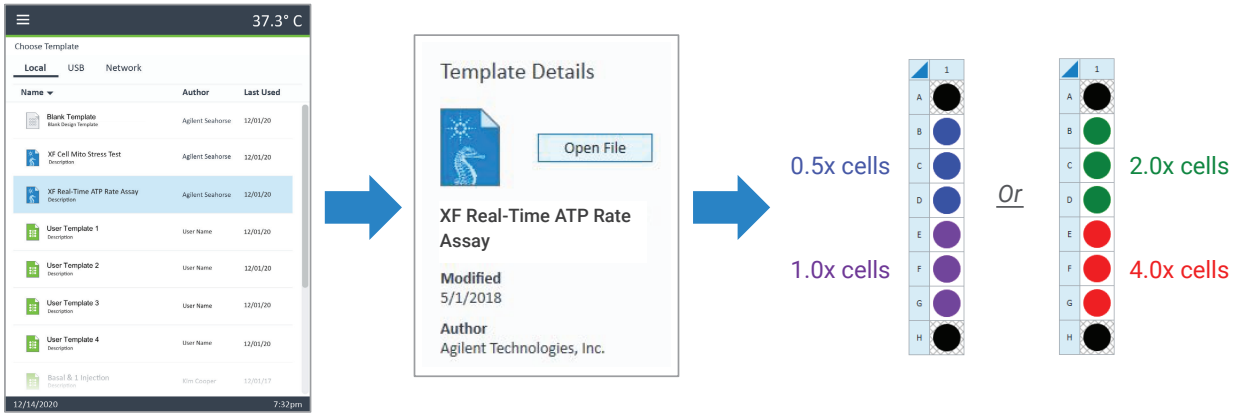

Figure 2. Select the XF Real-Time ATP rate assay template to modify.

9. Remove a hydrated XFp cartridge from the non-CO_2 incubator. Load each A and B 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

Injection Port volumes for XFp Real Time ATP Rate Assay Kit – Cell Characterization			
Port and 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 ports are filled, transfer the cartridge and utility plate to the XF HS Mini analyzer and begin cartridge calibration using the assay template created above.
11. Once cartridge calibration is complete, in the case of adherent cells, 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 XFp 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 Agilent Seahorse Analytics.

Further optimization of cell density

- In instances where the basal OCR values are *above* the recommended range of 20 to 150 pmol/min, then repeat the assay using an XFp cell culture plate seeded at a *lower* cell density.
- In instances where the basal OCR values are *below* the recommended range of 20 to 150 pmol/min, then repeat the assay using an XFp cell culture plate seeded at a *higher* cell density if the well surface allows it.
- Repeat data analysis as described above to determine if the basal OCR is now in the recommended range.

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