

## Cell Characterization: The XFe24 Analyzer and the XF Real-Time ATP Rate Assay



To effectively examine metabolic and bioenergetic function using the Agilent Seahorse XFe24 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 XF Real-Time ATP Rate Assay can be used to characterize the cell line/type of interest in a single assay.

A key parameter which must be empirically determined to properly characterize cellular metabolic function is the cell seeding density. Completion of this experiment provides an initial assessment of both 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  $1 \times 10^4$  and  $8 \times 10^4$  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](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 XF24 cell culture plate and an XFe24 cartridge and the XF Real-Time ATP Rate Assay with an XFe24 instrument.

### Day before Assay: 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.  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $4 \times 10^4$ ,  $8 \times 10^4$  cells/well).

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

	1	2	3	4	5	6
0.5X cells						
1.0X cells						
2.0X cells						
4.0X cells						

2. For each cell density to be tested, seed as directed for either adherent or suspension cells<sup>1</sup>.  
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](http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_BEFORE_CELL_SEEDING.pdf) or  
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 sensor cartridge the day prior to the XF assay: [http://www.agilent.com/cs/library/usermanuals/public/XFe24\\_DAY\\_BEFORE\\_CARTRIDGE\\_HYDRATION.pdf](http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf)

### Day of Assay

1. Prepare Seahorse 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 <sup>a</sup> / 103575-100 or Seahorse XF RPMI Medium, pH 7.4 <sup>a</sup> / 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

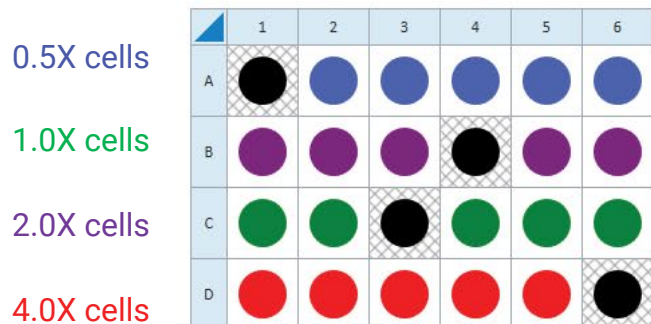
<sup>a</sup>XF DMEM and RPMI Medium, pH 7.4 have a pre-adjusted pH value and do not require adjustment of pH upon addition of XF supplements. See method below for preparation.

See: [http://www.agilent.com/cs/library/usermanuals/public/XFe24\\_DAY\\_OF\\_MEDIA\\_PREP.pdf](http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_OF_MEDIA_PREP.pdf)

2. 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 Seahorse XF Real-Time ATP Rate Assay Medium. Final well volume is 500  $\mu$ L. [http://www.agilent.com/cs/library/usermanuals/public/XFe24\\_DAY\\_OF\\_WASHING\\_CELLS.pdf](http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_OF_WASHING_CELLS.pdf)
5. Place the plate in a 37°C incubator without CO<sub>2</sub> for one hour prior to the assay.

<sup>1</sup>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.

6. Design an assay template in the Wave software by opening the XF Real-Time ATP Rate Assay Template. Below is a suggested plate map for testing the four different cell densities seeded:



7. 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

8. Remove the hydrated cartridge from the non-CO<sub>2</sub> incubator. Load each A and B ports of the XFe24 cartridge as outlined below and described at: [http://www.agilent.com/cs/library/usermanuals/public/XFe24\\_DAY\\_OF\\_LOADING\\_CARTRIDGE.pdf](http://www.agilent.com/cs/library/usermanuals/public/XFe24_DAY_OF_LOADING_CARTRIDGE.pdf)

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

Injection Port volumes for XF Real Time ATP Rate Assay Kit - Cell Characterization			
Port & Compound	Volume	10X [Port]	[Final Well]
Port A Oligomycin	55 µl	15 µM	1.5 µM
Port B Rotenone + Antimycin A	62 µl	5 µM	0.5 µM

9. Once all required ports are filled, transfer the cartridge and utility plate to the XFe24 instrument and begin cartridge calibration using the assay template created in step 6 above.

10. Once cartridge calibration is complete, wash the cells again with XF Real-Time ATP Rate Assay Media (final volume 500 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.

11. 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.

[www.agilent.com/chem/discoverxf](http://www.agilent.com/chem/discoverxf)

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**Not for use in diagnostic procedures.**

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