Seahorse Assay Overview Strategy (XFe96 well plate with green sensor plate)

Day before experiment:
Culture cells on Cell plate.
Preincubate sensor plate in H2O, 200ul / well.

Day of experiment:

1. Wash cells and add "minimal medium" to them and incubate for 90 min** in humid chamber at 37'C **without CO2**. **Do not let cells dry.** Cells need to be covered in liquid at all times.
   (= Medium that is bicarbonate free, hepes free, low in buffer capacity, and phenol red free)
2. Add calibrant to wells of sensor plate, 200 ul / well.
3. Wave software should be setup with your experiment ready to run.
4. Load drugs onto plate that have been diluted in Minimal Medium (make sure drugs don't have residual buffering medium).
   Use Guide plates to load drugs if helpful (these block the other drug ports when pipetting). Drugs must be added to each well port on the plate (usually, 20, 22, 25, 28 uL in ports A, B, C, D)
5. **REMOVE LID** and add sensor plate with the drugs to machine, wait 10 min to re-warm up.
6. Run Assay. This will first calibrate the sensor plate for 20 min.
7. When calibration is finished,
8. **REMOVE CELL CULTURE LID** and load your cell culture plate into the XFe96 machine.
9. Continue with Assay.

** Cells need to be happy in "minimal medium" during the duration of pre-incubation and the experiment. You can reduce pre-incubation time or use Seahorse XF assay media if your cells are sensitive. Happy cells = baseline OCR should be flat, drugs should have good effect.

Normalization
The OCR (Oxygen Consumption Rate) and ECAR (Extra Cell Acidification Rate) are dependent on the number of cells and therefore if the cell number is different between wells, you will want to normalize the final data (divide the reading) by the cell number.

Normalization suggestions

1. Imaging. Staining with Hoechst or other live cell dyes that stain nuclei. You can include this as well port D.
   Image the wells.
   Count the cells per well using QuPath or ImageJ.
   If your cells are well resolved on bright field, you may be able to count without staining the nuclei.
2. Colorimetric assay, such as BSA assay.
3. One of the fancy methods suggested by Aigilent.

JMW minimal medium =
145 mM NaCl, 2.5 mM KCL, 1 mM MgCl2, 1.8 mM CaCl2, 0.91 mM NaH2PO4, 2 mM glutamine (glutamax is not recommended), pH 7.4 with NaOH. Mitochondria stress test assay buffer additionally contained 10 mM glucose, 1 mM Na pyruvate. Glycolytic stress test buffer cannot contain glucose.

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Additional Notes

We have 2 Seahorse units: XFe96 (96-well plates) and XFp (8-well plates). I recommend the 96-well plates as replicates will reduce the standard error that comes with variations in cell density and other factors.

As of 2024-10-30 the price list for plates is here:
https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-sensor-cartridges-cell-culture-microplates/seahorse-fluxpaks-740883

For the XFe96
Cat. # 103792-100 : 18, 96-well plate systems. Price: $1479.00 = $82.16 per 96 well plate.
Seahorse XFe96/XF Pro FluxPak includes 18 XFe96/XF Pro sensor cartridges,18 XFe96/XF Pro Cell Culture Microplates, and 1 bottle of Seahorse XF Calibrant Solution 500mL.
Cat # 103793-100 : 6, 96-well plate systems. Price: $617 = $102.83 per 96 well plate.

For the XFp
Cat. # 103022-100 : 12, 8-well plate systems. Price: $558.00 = $46.5 per 8 well plate.
FluxPak includes 12 XFp sensor cartridges, 12 Seahorse XFp Cell Culture Miniplates, and Seahorse XF Calibrant Solution 100mL.

Suggested Sigma Aldrich drug sources (~Readnower 2012)

oligomycin	(Sigma, 75351)
FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone)	(Sigma, C2920),
antimycin A	(Sigma, A6874)
DMSO	(Sigma, 154938)
Rotenone	(Sigma, No Cat #)

1000X stocks in DMSO, Single use aliquots in frost free -20'C or -80'C freezer.
1000X concentration: 1 mM Oligomycin, 1 mM FCCP, 1 mM Rotenone and 1 mM Antimycin A.

Concentrations of drugs used by Murray et al (Huh-7 cells)
1 uM Oligomycin, 2 uM FCCP (uncoupler), 0.5 uM Rotenone.
10 mM glucose, 1 uM Oligomycin + 50 mM 2-Deoxyglucose (2-DG)

References
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Measuring Bioenergetics in T Cells Using a Seahorse Extracellular Flux Analyzer
Gerritje J W van der Windt , Chih-Hao Chang , Erika L Pearce
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