FCCP Optimization with the XF Cell Mito Stress Test

(Note: For this assay, seed cells at the optimal cell number and use the optimal oligomycin concentration that was determined in Training Assay 1.)

The XF Cell Mito Stress Test is run with six different concentrations of FCCP to determine the optimal FCCP concentration to use in your XF assays. In a typical FCCP optimization assay, it is not necessary to inject rotenone and antimycin A. However, for the purposes of providing richer data for discussion, we will run the XF Cell Mito Stress Test and inject (A) oligomycin (optimal concentration), (B) FCCP (six different concentrations) and (C) rotenone/antimycin A.

Plate Layout:

Injections:

All compounds will be made at 10x the final concentrations in the wells.

Port A: oligomycin: _____ µM (optimal) final concentration in the well

Port B: FCCP
Columns 1-2: 0 µM final concentration in the well
Columns 3-4: 0.125 µM final concentration in the well
Columns 5-6: 0.25 µM final concentration in the well
Columns 7-8: 0.50 µM final concentration in the well
Columns 9-10: 1.0 µM final concentration in the well
Columns 11-12: 2.0 µM final concentration in the well

Port C: rotenone/antimycin A: 0.5 µM final concentration in the well

Protocol:

1. Warm the pre-made XF Cell Mito Stress Test Assay Medium to 37°C.
   Adjust pH to 7.4 ± 0.1 at 37°C.
2. Retrieve your cell plate from the CO_2 incubator. Note the time.
3. Look at cells under the microscope to:
   a. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
   b. Ensure cells are adhered, and no gaps are present.
   c. Make sure no cells were plated in the background correction wells.
4. Wash cells with XF Cell Mito Stress Test Assay Medium
   a. Using a XF Prep Station
      i. Attach bottle of XF Cell Mito Stress Test Medium to XF Prep Station. Open the Seahorse XF Prep Station software. On the "Media Change" tab, select "Do Prime", set final volume to 180 µL of assay medium, and unselect "Do Rinse".
      ii. Place the cell plate vertically onto the tray and remove the lid.
      iii. Press "Start".
   b. Without using a XF Prep Station
      i. Remove all but 20 µL of the culture medium from each well.
      ii. Rinse cells two times with 200 µL of assay medium.
      iii. Add assay medium to each well for a final volume of 180 µL/well.
5. Look at cells under the microscope to ensure that cells were not washed away.
6. Place the plate in a 37°C incubator **without CO_2** for one hour prior to the assay.
7. Prepare Stock Compounds
   a. ***Important:*** *Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.*
   b. Remove foil pouch from XF Cell Mito Stress Test Kit box. Each pouch contains reagents sufficient for a complete XF Cell Mito Stress Test in a 96 or 24 well XF Cell Culture Microplate.
   c. Allow compounds to warm to room temp in the sealed pouch for approximately 15 minutes.
   d. Open pouch and remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap). Place tubes in a small tube rack.
   e. Resuspend contents of each tube with prepared assay medium in volumes described in table below with a p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds.

	Volume of Assay Medium	Final Concentration
Oligomycin	630 µL	100 µM
FCCP	720 µL	100 µM
Rotenone / AntimycinA	540 µL	50 µM

8. Prepare your compounds that you will load into the cartridge ports.

a. Prepare 3 mL of oligomycin in assay medium to achieve the desired final concentration determined in Training experiment 1 (or use 1 µM, which works for most cell types).

Port A Oligomycin	[Final well] (µM)	Stock volume (µL)	Medium volume (µL)
	0.5	150	2,850
	1.0	300	2,700
	2.0	600	2,400

b. Prepare serial dilutions of FCCP in assay medium, as detailed below.

Port B FCCP	Tube	[Final well] (µM)	Stock volume (µL)	Medium volume (µL)
	a	2.0	700	2,800
	b	1.0	1500 from a	1500
	c	0.5	1500 from b	1500
	d	0.25	1500 from c	1500
	e	0.125	1000 from d	1000
	f	0	0	2000

c. Pipette 300 µL of the rotenone/antimycin A stock into a 2700 µL aliquot of assay medium.

9. Get a hydrated cartridge from the non-CO_2 incubator. Load the cartridge in each port as outlined below.
   a. Port A – ____ µM oligomycin final concentration in the well. **Load 20 µL** of your 10x stock into each Port A.
   b. Port B – FCCP dilutions: **Note the layout! Load 22 µL** of each 10x solution into the B ports in the appropriate columns shown below.
      i. Columns 1-2: 0 µM final concentration in the well
      ii. Columns 3-4: 0.125 µM final concentration in the well
      iii. Columns 5-6: 0.25 µM final concentration in the well
      iv. Columns 7-8: 0.50 µM final concentration in the well
      v. Columns 9-10: 1.0 µM final concentration in the well
      vi. Columns 11-12: 2.0 µM final concentration in the well
   c. Port C – 0.5 µM Rot/AA final concentration in the well. **Load 25 µL** of your 10x stock into each Port C.
10. Create or load your assay template on the XF Controller. Default Mix-Wait-Measure times are 3 min – 0 min – 3 min. Usually 3 basal rate measurements are taken prior to the first injection; then 3 rate measurements after each injection.

11. On the Run Screen, Press Start and load the cartridge.
12. When prompted by the software, replace the Utility Plate with the Cell plate. Press Continue.