FCCP Optimization using the Cell Mito Stress Test with the Agilent Seahorse XF^{e}96/XF96

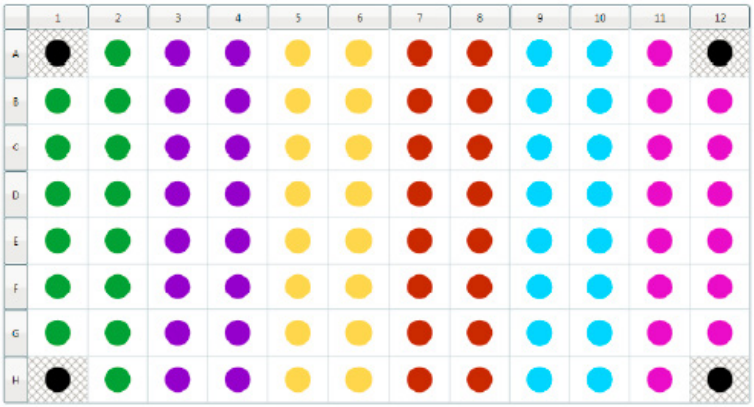
Basic Procedure

NOTE: For this assay, seed cells at the optimal cell number.

The Cell Mito Stress Test is run with six different concentrations of FCCP to determine the optimal FCCP concentration to use in your XF assays.

Plate Layout

[FCCP]	0 µM	0.125 µM	0.25 µM	0.5 µM	1.0 µM	2.0 µM



Injections

NOTE: All compound injection solutions are to be made at 10x their final respective concentrations in the wells.

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Port A: oligomycin

Port A: oligomycin: 1.0 µM (optimal) final concentration in the well

Port B: FCCP

1. Columns 1-2: 0 µM final concentration in the well
2. Columns 3-4: 0.125 µM final concentration in the well
3. Columns 5-6: 0.25 µM final concentration in the well
4. Columns 7-8: 0.50 µM final concentration in the well
5. Columns 9-10: 1.0 µM final concentration in the well
6. 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 Cell Mito Stress Test Assay Medium to 37˚C. For information on assay media preparation, see: http://www.agilent.com/cs/library/usermanuals/public/XFe96_DAY_OF_MEDIA_PREP.pdf and http://www.agilent.com/cs/library/usermanuals/public/XF96_DAY_OF_MEDIA_PREP.pdf. Adjust pH to 7.4 ± 0.1 at 37˚C.
2. Retrieve the cell plate from the CO_2 incubator.
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 show a consistent monolayer.
   c. Make sure no cells were plated in the background correction wells.
4. Wash cells two times with Cell Mito Stress Test Assay Medium, for detailed instructions see: 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. Look at 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 45-60 min prior to the assay.
7. Prepare Stock Compounds for Injection.
   a. Important: Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.
   b. Remove one foil pouch from XF Cell Mito Stress Test Kit box.
   c. Remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap).

d. Resuspend contents of each tube with assay medium using volumes described in the table below with a p1000 pipette to make Stock solutions for each compound. Gently pipette up and down (~10 times) or vortex with cap on to solubilize the compounds.

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

8. Prepare the compound solutions that will be loaded into the cartridge injection ports.
   a. Prepare 3 mL of 10 µM oligomycin in assay medium:

Port A Oligomycin	[Final well] (µM)	Stock volume (µL)	Medium volume (µL)
	1.0	300	2,700

   b. Prepare serial dilutions of FCCP in assay medium, starting with Tube a as detailed below:

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

Port C Rotenone/Antimycin A	[Final well] (µM)	Stock volume (µL)	Medium volume (µL)
	0.5	300	2,700

9. Obtain a hydrated cartridge from the non-CO_2 incubator. Load the cartridge in each port as outlined below.
   a. Port A – 1.0 µM oligomycin final concentration in the well. **Load 20 µL** of the 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 20 µL** of the 10x stock into each Port C.

10. Create or load your assay design on the XF^{e}96/XF96 Controller. Default Mix-Wait-Measure times are 3 min – 2 min – 3 min. In general, 3 basal rate measurements are taken prior to the first injection; then 3 rate measurements after each injection.
11. When ready, initiate the cartridge calibration and follow the screen prompts and load the cartridge.
12. After cartridge calibration is complete, follow the software prompts to replace the Utility Plate with the cell culture and initiate the assay.

Learn more
www.agilent.com/en-us/promotions/seahorse-xf-technology

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