

Oligomycin Optimization with the XF Cell Mito Stress Test

(Note: For this assay, seed cells at the optimal cell number that was determined previously.)

The XF Cell Mito Stress Test is run with four different concentrations of oligomycin to determine the optimal oligomycin concentration to use in your XF assays. In a typical oligomycin optimization assay, only three basal rate measurements followed by the oligomycin injection and three more rate measurements need to be taken to determine the optimal concentration of oligomycin. However, for the purposes of providing richer data for discussion, we will run the XF Cell Mito Stress Test and inject (A) oligomycin (4 concentrations), (B) FCCP and (C) rotenone/antimycin A. In this experiment, we will use 0.5 μM FCCP, although the optimal concentration of FCCP needs to be determined in a future assay.

Plate Layout:

	1	2	3	4	5	6	<u>[Oligomycin] (μM)</u>
A							0
B							0.5
C							1.0
D							2.0

Injections:

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

Port A: oligomycin

- Row A : 0 μM final concentration in the well
- Row B: 0.5 μM final concentration in the well
- Row C: 1.0 μM final concentration in the well
- Row D: 2.0 μM final concentration in the well

Port B: FCCP – 0.5 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 CO₂ 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. Remove all but 50 µL of the culture medium from each well.
 - b. Rinse cells two times with 1 mL of assay medium.
 - c. Add assay medium to each well for a final volume of 500 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** CO₂ 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 / Antimycin A	540 µL	50 µM

8. Prepare your compounds that you will load into the cartridge ports.
 - a. Prepare serial dilutions of oligomycin in assay medium, as detailed below.

Port A Oligomycin	Tube	[Final well] (µM)	Stock volume (µL)	Medium volume (µL)
	a	2.0	600	2400
	b	1.0	1500 from a	1500
	c	0.5	1000 from b	1500
	d	0	0	2000

- b. Combine 150 μL of the FCCP stock with 2850 μL of assay medium.
 - c. Combine 300 μL of the rotenone/antimycin A stock with 2700 μL of assay medium.
9. Get a hydrated cartridge from the non-CO₂ incubator. Load the cartridge in each port as outlined below.
 - a. Port A – oligomycin dilutions: **Note the layout! Load 56 μL** of each 10x solution into the A ports in the appropriate rows shown below.
 - i. Row A: 0 μM final concentration in the well
 - ii. Row B: 0.5 μM final concentration in the well
 - iii. Row C: 1.0 μM final concentration in the well
 - iv. Row D: 2.0 μM final concentration in the well
 - b. Port B – 0.5 μM FCCP final concentration in the well. **Load 62 μL** of each 10x solution into each Port B.
 - c. Port C – 0.5 μM Rot/AA final concentration in the well. **Load 69 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 – 2 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.