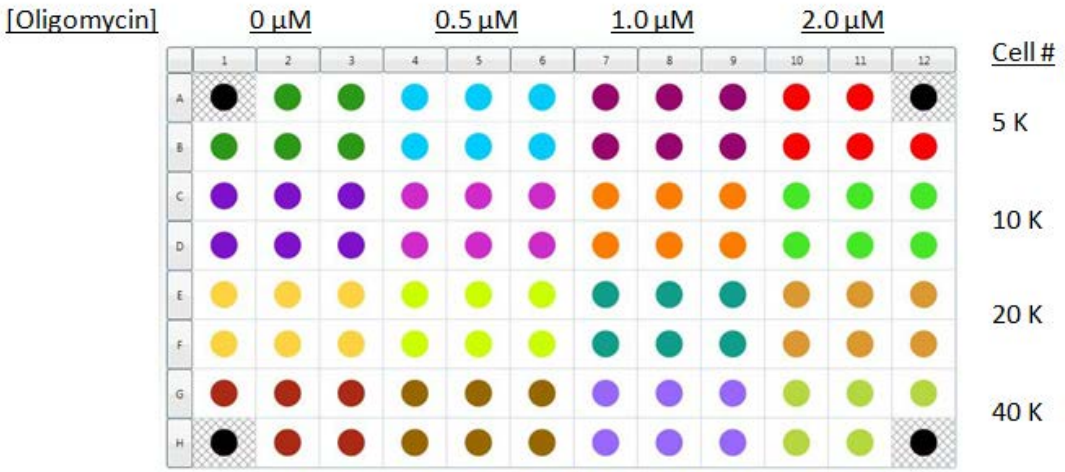
Cell Density and Oligomycin Optimization with the XF Glycolysis Stress Test

This procedure describes using the XF Glycolysis Stress Test with four different cell densities and four different concentrations of oligomycin to determine the optimal cell density and oligomycin concentration to use in XF assays. In a typical cell density and oligomycin optimization assay, only three basal rate measurements followed by the oligomycin injection and three more rate measurements, are needed to determine the optimal cell seeding density and the optimal concentration of oligomycin. However, for the purposes of providing richer data for discussion, we will run the XF Glycolysis Stress Test and inject (A) Glucose, (B) Oligomycin (4 concentrations) and (C) 2-Deoxy-D-glucose (2-DG).

Plate Layout:



Injections:

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

Port A: Glucose - 10 mM final concentration in the well

Port B: Oligomycin

Columns 1-3: 0 µM final concentration in the well
Columns 4-6: 0.5 µM final concentration in the well
Columns 7-9: 1.0 µM final concentration in the well
Columns 10-12: 2.0 µM final concentration in the well

Port C: 2-DG – 50 mM final concentration in the well

Protocol:

1. Warm the pre-made XF Glycolysis 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:
   i. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
   ii. Ensure cells are adhered, and no gaps are present.
   iii. Make sure no cells were plated in the background correction wells.
4. Wash cells with XF Glycolysis Stress Test Assay Medium
   i. Using a XF Prep Station
      a. Attach bottle of XF Glycolysis 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".
      b. Place the cell plate vertically onto the tray and remove the lid.
      c. Press "Start".
   ii. Without using a XF Prep Station
      a. Remove all but 20 µL of the culture medium from each well.
      b. Rinse cells two times with 200 µL of assay medium.
      c. 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 the stock compounds from the XF Glycolysis Stress Test (*For more details, refer to the XF Glycolysis Stress Test User Guide).*
   i. ***Important:*** *Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.*
   ii. The XF Glycolysis Stress Test Kit includes 6 foil pouches each containing oligomycin, 6 vials containing glucose and 6 vials containing 2-DG. The kit reagents are sufficient for 6 complete XF Glycolysis Stress Test assays in a 96 or 24-well XF Cell Culture Microplate.
   iii. Remove one foil pouch containing oligomycin and 1 vial containing glucose and 1 vial containing 2-DG from the kit box.
   iv. Allow compounds to warm to room temperature in the sealed pouch/vials for approximately 15 minutes.
   v. Re-suspend each component with prepared assay medium in volumes described in Table 3 with a p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds. Vortex the 2-DG for at least 1 minutes to ensure that it goes into solution.

	Volume of Assay Medium	Final Concentration
Glucose	3000 µL	100 mM
Oligomycin	720 µL	100 µM
2-DG	3000 µL	500 mM

8. 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	1000
	d	0	0	2000

9. Get a hydrated cartridge from the non-CO_2 incubator. Load the cartridge as outlined below.
    a. Port A – 10 mM Glucose final concentration in the well. **Load 20 µL** of the 10x solution into each Port A.
    b. Port B – Oligomycin dilutions: **Note the layout! Load 22 µL** of the 10x solutions into each Port B according to the plan.
        1. Columns 1-3: 0 µM final concentration in the well
        2. Columns 4-6: 0.5 µM final concentration in the well
        3. Columns 7-9: 1.0 µM final concentration in the well
        4. Columns 10-12: 2.0 µM final concentration in the well
    c. Port C – 50 mM 2-DG final concentration in the well. **Load 25 µL** of the 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.