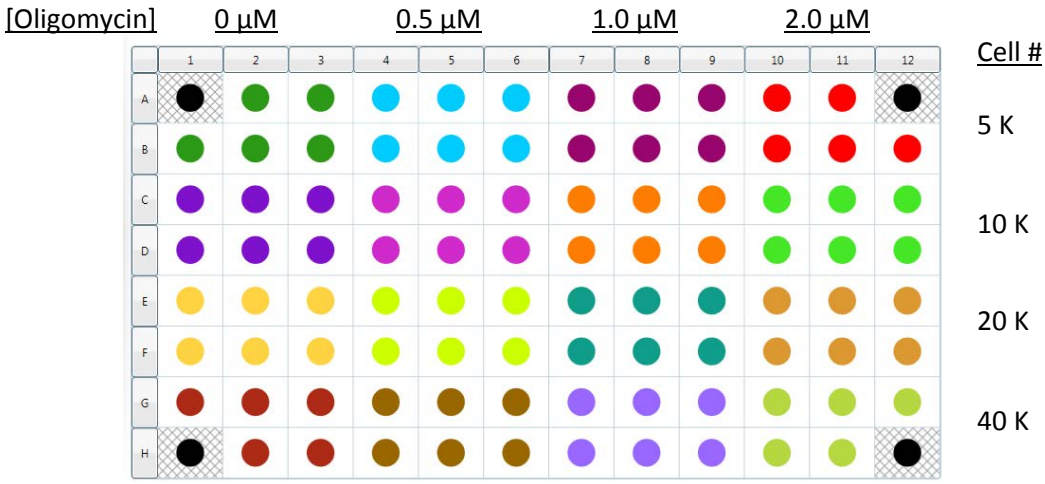
Cell Density and Oligomycin Optimization with XF Glycolysis Stress Test

The XF Glycolysis Stress Test is run with four different cell densities and four different concentrations of oligomycin to determine the optimal cell density and oligomycin concentration to use in your 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, need to be taken 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:

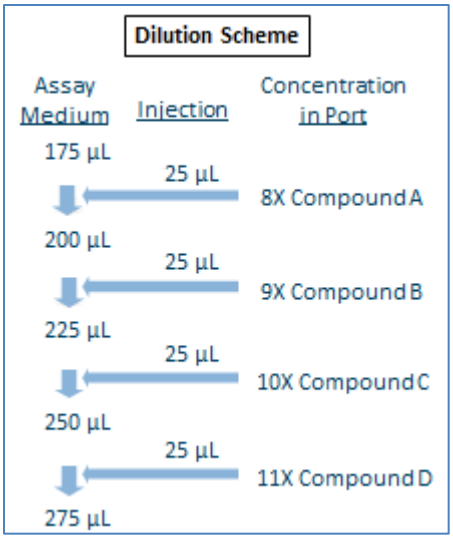


Injections:

Port A: Glucose - 10 mM final concentration in the well (80 mM stock)

Port B: Oligomycin

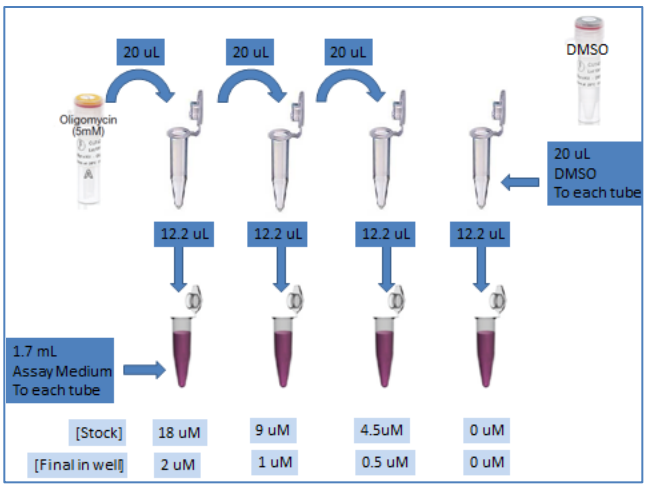
Columns 1-3: 0 µM final concentration in the well (0 µM stock)
Columns 4-6: 0.5 µM final concentration in the well (4.5 µM stock)
Columns 7-9: 1.0 µM final concentration in the well



(9 µM stock)
Columns 10-12: 2.0 µM final concentration in the well
(18 µM stock)

Port C: 2-DG – 100 mM final concentration in the well
(1 M stock)
Protocol:

1. Warm the pre-made Glycolysis Stress Test Assay Medium to 37°C. Adjust pH to 7.35 ± 0.05 at 37°C.
2. Thaw 1 set of vials from previously reconstituted XF Glycolysis Stress Test kit (glucose, oligomycin and 2-DG).
3. Retrieve your cell plate from the CO_2 incubator. Note the time.
4. 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.
5. Wash cells with XF Glycolysis Stress Test Assay Medium
    a. Using a XF Prep Station
        i. 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 175 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 155 µL of assay medium to each well for a final volume of 175 µL/well.
6. Look at cells under the microscope to ensure that cells were not washed away.
7. Place the plate in a 37°C incubator **without CO_2** for one hour prior to the assay.
8. Dilute the stock compounds from the XF Glycolysis Stress Test that you will load into the cartridge ports
    a. Pipette 96 µL of 2.5 M glucose into a 3 mL aliquot of assay medium.
    b. Prepare serial dilutions of oligomycin in DMSO, as shown below.
        i. Pipette 20 µL of DMSO into each tube.



ii. Pipette 20 µL of oligomycin into first tube. Mix.
iii. Perform two more serial dilutions as shown.
iv. Pipette 12.2 µL of each serial dilution into 1.7 mL aliquots of assay medium.

c. 1000 mM 2-DG is not diluted before loading.

9. Get a hydrated cartridge from the non-CO_2 incubator. Load the cartridge with 25 µL in each port as outlined below. **Note the layout!**
   a. Port A - 10 mM Glucose final concentration in the well (80 mM stock)
   b. Port B –Oligomycin dilutions: **Note the layout!**
      i. Columns 1-3: 0 µM final concentration in the well (0 µM stock)
      ii. Columns 4-6: 0.5 µM final concentration in the well (4.5 µM stock)
      iii. Columns 7-9: 1.0 µM final concentration in the well (9 µM stock)
      iv. Columns 10-12: 2.0 µM final concentration in the well (18 µM stock)
   c. Port C – 100 mM 2-DG final concentration in the well (1000 mM stock)

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.