Standard Glyco/Mito Stress Seahorse Assay

Night Before Seahorse Assay

1. Hydrate a sensor catridge. Must be done a minimum of 3 hours before running the assay.
    a. Place the Sensor Cartridge upside down next to the Utility Plate. Fill each well of the Utility Plate with 200 µL of Seahorse XF Calibrant. Lower the Sensor Cartridge onto the Utility Plate submerging the sensors in XF Calibrant.
2. Verify the XF Calibrant level is high enough to keep the sensors submerged. 6.
3. Place in a non-CO_2 37 °C incubator overnight. To prevent evaporation of the XF Calibrant, verify that the incubator is properly humidified.
4. If running adherent cells, plate these cells in R10/D10 and allow to sit at 37 °C overnight
    a. Cell numbers per well may vary between cell lines.
    *See Seahorse Cell Reference Sheet

Day of Seahorse Assay

1. "Cell Tak" an XF^{e}96 Cell Culture Microplate (for non-adherent cells).
    - 3mL 1X PBS
    - 7uL Cell Tak
    - 3.5uL NaOH

    Distribute 30 µL of mixture to wells of the cell culture microplate using a multichannel pipette. Allow plate to sit for at least 20 minutes. Aspirate liquid.

2. Mix up "Standard Mito Stress Test Assay Media", pH to 7.4 ± 0.05.

Reagent	Amount	Final Concentration
Seahorse XF Base Medium (Minimal DMEM)	98mL	N/A
L-Glutamine (200mM)	1mL	2mM
Sodium Pyruvate (100mM)	1mL	1mM
Glucose	180mg	10mM

3. Prepare inhibitors, each in 3 mL of assay media. Note- inhibitors are made at 10X concentrations so that they are 1X upon injection into the well. pH to 7.4 ± 0.05

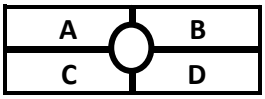
Injection Port	Inhibitor	Amount	Final Concentration
A	Oligomycin (5mM)	12uL	2uM
B	FCCP (5mM)	Varies	Varies between cell type *See Seahorse Cell Reference Sheet
C	2-deoxy-glucose (600mM)	500uL	10mM
D	Rotenone (2.5mM)	6uL	0.5uM

	Antimycin A (3mM)	5uL	0.5uM

4. If running suspension cells, spin cells down at 1500rpm for 5min. Resuspend in standard mito stress test media (180uL per well). Fill blank wells with 180uL media. Spin plate at 800rpm for 3min. After spin, check to see if cells are stuck to the bottom of the plate. Place in the non-CO2 37 °C incubator for 45min-1hr prior to run.
    a. Cell numbers per well will vary between cell types
    *See Seahorse Cell Reference Sheet

5. Before loading the cartridge, lift sensors up from calibrant a few times until all bubbles in the plate are gone.

6. Using the provided Seahorse loading guides, loading the inhibitors into the appropriate injection ports using a multichannel pipette.



Loading Scheme	
A	20 µL
B	22 µL
C	25 µL
D	27 µL

Notes:

- Double check that you are using the appropriate loading guide for the appropriate injection port.
- Double check that the blue triangle is in the bottom left-hand corner of the cartridge before loading any ports.
- Be sure to change the pipette volume each time you switch inhibitors! This is very easy to forget to do.
- When using the loading guides, check for residues leftover on the loading guide after loading one port and before loading the next. You do not want to transfer one inhibitor into another port by accident!

Protocol for T cell anti-CD3/anti-CD28 Activation in Seahorse

Night Before Seahorse Assay

- Follow steps 1-3 in glyco/mito test

Day of Seahorse Assay

- Steps 1-4 are relatively the same, but see below for media recipe and injections

Standard Activation Media

Reagent	Amount	Final Concentration
Seahorse XF Base Medium (Minimal DMEM)	99mL	N/A
L-Glutamine (200mM)	1mL	2mM
Glucose	180mg	10mM

Injections

Injection Port	Activator/Inhibitor	Amount	Final Concentration
A	1mg/mL Streptavidin, 0.5mg/mL anti-CD3, 1mg/mL anti-CD28	45uL 180uL 60uL	1.5ug/mL 3ug/mL 2ug/mL
B	DCA (1M)	600uL	20mM
C	2-deoxy-glucose (600mM)	500uL	10mM

Notes:

- When working with PA cells, make sure to rest the cells in before activation.
    - Do not use cells earlier than day 7
    - When activating cells, culture in 50U/mL IL-2 for 2 days, 25U/mL IL-2 for 4 days, and then rest in 10U/mL IL-2 for 1-2 days (rest in 10U/mL night before the assay)
- Allow streptavidin to crosslink for at least 15min at 37 °C before activation.

Protocol for Glycolysis Stress Test

Night Before Seahorse Assay

- Follow steps 1-3 in glyco/mito test

Day of Seahorse Assay

- Steps 1-4 are relatively the same, but see below for media recipe and injections

Glycolysis Stress Test Media

Reagent	Amount	Final Concentration
Seahorse XF Base Medium (Minimal DMEM)	99mL	N/A
L-Glutamine (200mM)	1mL	2mM

Injections

Injection Port	Activator/Inhibitor	Amount	Final Concentration
A	Glucose	54mg	10mM
B	Oligomycin (5mM)	12uL	2uM
C	2-deoxy-glucose (600mM)	500uL	10mM