Imaging Facility
Biotechnology Resource Center
Cornell University
BRC_Imaging@cornell.edu

# User Guide: Agilent Seahorse Units (B38 Weill)

For ordering purposes, the Seahorse-96 is called the XFe96 and the Seahorse-8 is the XFp. These instruments measure oxygen consumption and pH in the extracellular solution in a small, defined region around cells attached to the bottoms of specialized 96- or 8- cell culture plates.

## What do you need for this assay?

1. A plan. Come talk to us if you need help with this.
2. A Seahorse sensor plate, a Seahorse cell culture plate and Seahorse calibrant solution. You can either buy these in bulk (FluxPaks) or buy "singles" from us.
3. Bicarbonate-free (BF) media. (Phenol red may also cause problems, but this is not as critical.)
4. Specific inhibitors or drugs for injecting. Agilent sells specific combinations of drugs – "assay kits" or "test kits" – to probe specific pathways. These test kits are embedded into the software, making analysis more automated. You can find a summary of Seahorse assay kits here:

   https://www.agilent.com/cs/library/flyers/public/5991-8291EN.pdf
5. Use Poly-D-Lysine if you have non-adherent cells.

## Need more information?

General info:

https://www.agilent.com/search/?N=135&Ntt=seahorse

Seahorse-96 (XFe96) Consumables:

https://www.agilent.com/cs/library/quickreference/public/XFe96_KYS.pdf

Seahorse-8 (XFp) Consumables:

https://www.agilent.com/cs/library/quickreference/public/XFp_KYS.pdf

****All items available on Cornell Eshop. Search Agilent and product number.*

## Consumables available for purchase in the facility

| Item | Description |
|---|---|
| Seahorse-96 (XFe96) fluxpak single | One 96-well sensor plate<br>One 96-well cell culture plate (and an extra if you want one -- please only take if you need)<br>20 ml aliquot of XF calibrant |
| Seahorse-8 (XFp) fluxpak single | One 8-well sensor plate<br>One 8-well cell culture plate<br>XF calibrant as needed |
| Seahorse XF base medium | 25 ml aliquot of DMEM without sodium bicarbonate, glucose, glutamine/GlutaMAX or sodium pyruvate |

## The day before

1. Pre-incubate sensor plate overnight in sterile water (200 µl/well) in a non-$CO_2$ incubator at 37ºC. Agilent alternatively refers to the sensor plate as the "sensor cartridge" and the "utility plate".

2a. For adherent cells, plate 10-30K cells/well in the Seahorse cell culture plate in their standard media (200 µl/well) and let sit overnight in whatever incubator you normally use. (This step can also be done the same day.)

2b. For non-adherent cells, coat Seahorse cell culture plate with Poly-D-Lysine (see Appendix A: Example XFp Protocol for T-cells)

## Assay prep (an hour before)

1. Warm all media and compounds to 37ºC and ensure a correct pH of 7.4.

2. For the sensor plate, remove water and replace with calibrant solution (200 µl/well).

3a. For adherent cells, replace standard media with bicarbonate-free (BF) media (200 µl/well), and allow cells to equilibrate in non-$CO_2$ incubator.

3b. For non-adherent cells, re-suspend cells in BF media, and spin them (gently) onto the Agilent cell culture plate (10-30K/well). Allow cells to equilibrate in non-$CO_2$ incubator. See Appendix A: Example XFp Protocol for T-cells.

4. Start the "Wave" software. Load XF Assay template into the software or make a new template using the Assay Wizard. Use the table below to program the optimal mix, wait and measure cycle times. When working with a new or unknown cell line, start with a 3 minute mix and 3 minute measure cycle.

5. Load 20, 22, 24, and 26 µl of pre-warmed compounds (diluted in BF media) into A, B, C and D ports on the sensor cartridge for 1/10 dilutions. All wells of each port used need to be loaded, including background wells. (For example, if you are injecting with port A, all the A ports need to be filled with something.) If the sensor cartridge is allowed to cool down (more than 5 minutes), return to incubator for 10 minutes to allow it to heat back up before starting the calibration.

## Running the assay

1. Load sensor plate into the instrument tray **without the lid.**

2. Press the run assay button.

3. After calibration is complete, the software will prompt you to replace the utility plate with the pre-incubated cell plate. **Remove the lid first!**

# Appendix A:  Example XFp Protocol for T-cells

(Provided by Cybelle Tabilas, Brian Rudd's lab)

### *Mito Stress Kit Buffer: PH=7.4*

- Seahorse XF base Medium Minimal DMEM
- 20 mM glucose
- 2 mM L-glutamine
- 1 mM Sodium Pyruvate
- For 100 ml media, need 0.36 g glucose, 1 ml stock L-glutamine solution and 1 ml Sodium Pyruvate solution (calculated based on our current stock)
- Current stock: glucose MW: 180.16 g/mol, L-glutamine: 200 mM and Sodium Pyruvate: 100 mM

### *Glycolysis Stress Kit Buffer: PH=7.4*

- Seahorse XF base Medium Minimal DMEM
- 2 mM L-glutamine

### *PDL solution:*

- Poly-D-Lysine (Sigma) to attach semi-adhesive T cells (PDL)
- Make working solution at 50ug/ml with sterile water

### *Coating:*

1. In the hood, add 50 ul per well to XFp plate.
2. Incubate at room temperature for 1-2 hours.
3. Aspirate the solution.
4. Rinse the surface with 200 ul sterile water and aspirate off.
5. Allow the surface to dry with lid open in the hood.
6. Wrap coated plates and store them at 4°C fridge and allow them to warm up to room temperature before running assay.

## One day prior to assay:

1. Hydrate the XF sensor cartridge.
2. Add 200uls of sterile H2O to each well and 400 uls to each moat of the Xfp cartridge.
3. Place the XF Sensor Cartridge on top of the utility plate and place in a 37°C incubator **without $CO_2$** for 12-72 hours.

4. Day of or day before: pH media in Aguilar or Russell lab (pH day of and put in 37º without $C0_2$ incubator to avoid pH change).

## What to bring to BRC seahorse:

- P1000 + P200 + appropriate tips
- Old pipette tip with a heating pack for medium
- Appropriate media

## Plating cells:

1. Harvest cells.
2. Replace $H_2O$ in the cartridge with 200 uls of Xf calibrant to each well and 400 uls into each moat.
    a. This should be done about 45 minutes before loading drugs.
    b. This helps with dehydration (mostly for humid areas in the country).
3. Spin down cells at 300 g for 5 minutes; set rotor to R12/15 accel/decel N/N.
4. Resuspend 300,000 cells/50 ul (6e6/ml) in desired XF assay buffer.
    a. (# cells * 50)/300k = amount to re-suspend cells in with appropriate media
5. Plate 50ul of cell suspension per well on coated plate and 50ul media in blank wells.
6. Spin plate for 1 min at 300g, set rotor to R2 accel/decel S/0.
7. Check for an evenly distributed layer of cells using compound microscope.
8. Add 130 ul of XF assay buffer **slowly** to each well.
    a. Total media should be 180 uls
9. Re-check the layer of cells for even distribution; if disrupted, spin again.
10. Place plate in non-$CO_2$ incubator until loading and let seed for 1 hour (very important!).
11. During incubation, load drugs onto cartridges drug ports.
12. Put in cartridge first to calibrate, then cell plate (follow instructions on Seahorse).

## Drug concentrations and loading volumes for assays:

**Note:**

- All drugs concentrates are 10X of working concentration because once drugs are added onto cell plate, they will get diluted 10 fold with the media in the well.
- BE GENTLE WHEN LOADING AND HANDLING CARTRIDGE WITH DRUGS.

- *We use sigma Oligomycin (stock solutions are in -20C in DMSO).*

## Mitostress kit:

| | Volume of buffer to add (uL) | Final Concentration (uM) | Loading Concentration (uM) | Loading volumes (ul) |
|---|---|---|---|---|
| Oligomycin (A) | X (1:100) | 10 | 1 | 20 |
| FCCP ( B) | 288 uL | 10 | 1 | 22 |
| R/A ( C) | 216 ul | 5 | 0.5 | 25 |

## Glycolysis kit:

| | Volume of buffer to add (uL) | Final Concentration (uM) | Loading Concentration (uM) | Loading volumes (ul) |
|---|---|---|---|---|
| Glucose (A) | 300 uL | 100 mM | 10 mM | 20 |
| Oligomycin (B) | X (1:100) | 10 uM | 1 uM | 22 |
| 2DG © | 300ul | 500 mM | 50 mM | 25 |