PSBLAB – Advance Considerations for those planning to do Seahorse experiments

1) Two Seahorse XF systems are available – XF96 in PSBLAB, and XFe96 in core facilities. The XF96 is an older machine (2012) and is no longer covered by warranty or service contract, so may not be available long term. Thus, we advise to use XFe96 in core if you intend to do a large series of follow-up experiments.

2) Important first step is to ensure your cells are ADHERENT to the plastic of the XF plates. Plates are available in 2 types of plastic – PS and PET. Borrow a few plates from PSB lab and play around with plating conditions if needed. It may be necessary to coat plates with laminin/fibronectin or a reagent such as CellTak (Invitrogen) to get the cells to adhere.

3) Plating density should be sub-confluent (contact inhibition has effects on metabolism). Depending on cell type, number of cells per well can be anywhere from 2,000 to 100,000. For large metabolically active primary cells (cardiomyocytes, hepatocytes) 2-5k cells/well is usually OK. For cancer or transformed cells in culture, 10-50k, and for hematology (leukocytes, platelets) >100k/well.

4) Plate cells in a small volume, allow to settle by gravity (or spin in plate rotor), then top up media to fill well. Do not plate cells in whole volume, since then they will stick up the sides of the well. We have a 96-well plate rotor in the lab centrifuge if needed.

5) If using XF96 in PSBLAB, download software for XF96 by searching for "XF96 software" on Agilent website (no permanent link available, it should be available under "legacy software" link on the website, or get the .exe file from us). Make sure to choose this, and not XFe96 or any other software. If using XFe96 in core, download newer Seahorse "wave" software. Software is free to download, use, copy, etc., and only runs on PC. No Mac version. Software is not cross-compatible, so data or methods made in one cannot be viewed in the other.

6) Data and all other files are stored/transferred as Excel spreadsheets. If choosing to visualize data in Excel, you will need to downgrade security settings to run macros. On some older PCs, Adobe Flash plugin for Internet Explorer is required for the XF96 software to run properly. Typical data files are 12-15MB.

7) To book PSBLAB instrument, make a reservation by email a week in advance. If using the core XFe96, book via their calendar system (training is required to become a user).

8) The experimental set-up comprises the plate the cells are grown/seeded on, and a disposable cartridge that contains the O_2 and pH sensors, which sits on top. Plates are cheap – we can provide spares if needed. Cartridges are $55 each. They come in packs of 6 or 18 ("Flux-Paks"), with cell plates and the calibration solution. A typical series of experiments for a figure in a paper may use 6-10 cartridges. We can provide a MAXIMUM of two cartridges, for troubleshooting and getting experiments up and running. After this, you must buy your own. No *just-one-more* exceptions.

9) The night before the experiment, the cartridge has to be hydrated – pipet 100uL of hydration media into the "utility plate" that comes with it, then seal plate back in its container (or seal the edges with parafilm). Also now is a good idea to check that instrument is turned on so it can get up to temperature (37C), and to make your method using the software.

10) Typical experiment will add various inhibitors via the drug injection ports on the cartridge. Ports must be loaded with drugs before the cartridge is put into machine for calibration. Typical drug injection volumes are 15-25uL for the XF96. For example if starting out with 150uL of media in each well, then 25uL addition would be a 1/7 dilution (25uL in 175uL final). So, stock drug needs to be made up at 7x strength <u>in same media that</u>

measurements are made in. Max' volume is 200, so if using all 4 ports then suggest to start with 140uL and then do 4 x 15ul injections. Don't go below 15 uL or injections may not work properly.

As with cartridges, we can provide one aliquot of the standard drug mixtures (oligomycin, FCCP, antimycin, rotenone) for a first experiment, but no more. Suggest to buy the "mito stress test kit" from Agilent, which contains the drugs already aliquoted in correct amounts for a plate.

11) For the media, most experiments are done in an un-buffered version of DMEM (i.e. no bicarb) – this is to allow measurements of ECAR (extracellular acidification rate, proxy for glycolysis). There are versions that can be purchased directly from Agilent/Seahorse, or from Sigma. Best thing is to buy a glucose, glutamine, bicarb free DMEM, and then add back the things you need. A key consideration is what to add as fuel (metabolic substrate)… Glucose, fat, ketones, glutamine, lactate? It is also possible to add this as an experimental variable – i.e. put different fuels in different wells and see what the cells respire best on. Fat must be conjugated to BSA to work properly – Agilent sells it pre-conjugated, but we have a protocol to make it from scratch for a lot less $$. We cannot routinely provide media for experiments – it's variable depending on your question. You must determine things such as whether cells are happy without serum. Generally, there should be no phenol red in media as this can interfere with measurements.

12) The media the cells are grown in is usually NOT the same as the media for the experiment. Typically, cells must be switched to the experimental media 1 hr. prior to the experiment. Then the plate sits at 37C while the instrument calibration occurs.

13) The actual experiment will usually consist of 10-12 sets of OCR/ECAR measurements. The machine cycles through the plate every 15 seconds, so takes about 4 readings a minute, and it's recommended to read for at least 3-5 minutes initially, to get a good slope (O2 vs. time) in order to calculate the rates. Also there are wait times and mixes and injections, so overall an experiment will take about 80-120 min. Typical protocol would be:

(1) Calibrate – this is the cartridge sitting in calibrant, seeing if all the sensors are in-range.
(2) Equilibrate – this is the cartridge adjusting to being switched to your cell-media.
(3) Mix
(4) Measure
(5) Mix
(6) Measure
(7) Mix
(8) Measure
(9) Inject drug
(10) repeat steps 3-9, etc.

So, each condition (e.g., with or without drug) gets 3 mix/measure cycles. Exact measure time will depend on how fast the cells respire – you need long enough to get a good slope (pO_2 vs. time), but if cells respire too fast then they'll go hypoxic in the wells. The best solution is to watch the instrument for first couple of reads, and if necessary you can adjust the read times for the remainder of the protocol on-the-fly.

14) On day of experiment, workflow is roughly as follows…

(1) Make up stock solutions of drugs to be injected
(2) Replace media for experiment (use cell culture hood).
**Cells now in bicarb free media, must be kept at 37C but NOT in a CO_2 incubator.
(3) Load drugs into cartridge. A multi-channel pipettor is essential for this. Bring your own tips.
(4) Load method onto computer that controls the XF machine.

(5) Press start, load cartridge. Calibration takes 15-10 min.
(6) Machine will now eject base plate with calibration media – take this out and put in your cell plate.
** Cartridge stays in machine after calibration – don't strip or eject it, or you'll have to recalibrate.
(7) Equilibration (~10 min) then measurements. Can now leave machine to run.

15) Other notes.
Computer that runs the XF96 is not connected to the InterNet, so you'll need a USB stick to retrieve data.

The XF96 software has a known RAM leak, so if machines are left on for weeks at a time, this can result in a crash in the middle of a run. Best way to avoid this is reboot the PC when you come to the lab to do your experiment.

The method (specifically, measurement times) can be edited on-the-fly in the program while the assay is running. For this reason, it's a good idea to watch the first couple of measurements to make sure OCR values are reasonable and there's nothing else wrong. Once your mito toxins and other drugs are all injected there's no way to go back and undo it. When setting up a method, avoid the "loop" function – you can only edit a step in the protocol if it hasn't begun yet, so if you set up a loop of 3 measurements and you don't like the results after the first one, you have to wait until the whole loop of 3 is finished before you can edit. Even though individual steps mean a more cumbersome methods file, it's more flexible for troubleshooting during the run.

Cartridges and cell plates are bar-coded and become a matched pair when used together. Thus, cartridges cannot be re-used. It is not a good idea to try and re-use them anyway – they're almost impossible to wash out and clean all the drug ports, and the plastic might be porous to lipophilic mito' inhibitor drugs, which could carry over into your next assay. If you really want to save money, the only way would be to peel the bar-code off a cell plate after use, and then stick it on a different plate of cells and re-use the paired cartridge within the same day, but this is not very reliable (bar codes often mis-read). Also if you wash and re-use a cartridge, if you get water down into the probe holes, you could damage the machine since the rods holding the fiber-optics are made of steel and will rust. IF you only have a few cells, you can use half the plate, then use the other half of the same cell plate later on (cartridge is good for 3 days), BUT be aware the inside of the instrument is not sterile, so plating cells on an already-used plate carries a risk of contamination.

One problem we frequently see is people trying to fast forward the troubleshooting steps. There are essentially 3 steps to making sure you get reliable data … cell number, substrate, and drug titrations (e.g., for FCCP). You can try to skip this (e.g., by using a generic amount of FCCP) but then may run into problems later. Different cells will take up (and spit out) drugs at different speeds. Some cells may take longer to take up a drug, so you may need to add a few extra measurements after drug addition, to be sure it is taken up properly. Different cells obviously burn different fuels (heart burns fat, brain burns glucose, etc.). Your first plates should be dedicated to doing a cell-seeding titration and some drug titrations, to find the correct ranges. Also look up in Agilent's database to find publications where people have used the same cell type for Seahorse experiments.

You will need to leave at least 4 wells (preferably more) blank – no cells, media only. Usually these are the 4 corner wells plus a couple more in the center. In the XF96 it is common to leave 2 columns clear on the edges of the plate (column 1 and 12). Bonus - you can then use these empty wells for protein standards later on, if running a protein assay on the plate.

For drug injections, you can put different drugs in each port (A,B,C,D) but all ports should be loaded with something. In the program, the instruction to inject results in all of one port being injected, across the whole plate. So, typically if you only want to add drug to some wells, you need to load vehicle (media) in the other ports. This is the only way to inject half the plate with drug. You cannot address each well individually.

Another problem we see is people trying to fit too many experimental variables on a plate. The variation from well-to-well is pretty large, so we normally advise at least 5-6 wells per condition. This might seem like a lot, but if you have 2 wells that "don't look right" or are outliers, you can discard them and still have 4 remaining data points to get a mean +/- SE. If you only do 3 wells per condition, you're asking for trouble later on if one or more wells has a problem.