ANTIBODY TITRATION PROTOCOL

NOTE: When titrating an antibody for use in flow cytometry, you should attempt to titrate it under the same conditions in which it will be used during your experimental cell staining. This means using roughly the same number of cells per tube, same volume of stain, same length of time for staining, same method of washing, and choice of fixative (or lack thereof) as you will use in your experiment. This protocol is the method I have used in the past—you may modify things slightly to suit your protocol.

* Take note of the vendor, catalog number, and lot number of each antibody you're titrating. You should titrate each new vial of antibody you receive in your lab, even if you've used the same catalog number before. This becomes especially critical when using tandem conjugates (such as PE-Cy7, APC-Cy7, or PerCP-Cy5.5) which vary from lot to lot even from the same manufacturer.

* Prepare cells for staining – you'll probably want to use cells similar to what you'll stain experimentally. Obviously, you should know that the antibody you're titrating will have a good number of binding sites on these cells.

* Determine cell concentration.

* Pipet approximately 1 x 10^6 cells into each FACS tube (12 x 75 mm) for each titration dilution. You'll want 6 tubes for each Ab: one unstained, plus 5 dilutions. Keep on ice til ready to spin.

* Make serial dilutions of your antibody in Staining Medium (SM) as follows:

1:50 1:100 1:200 1:400 1:800
Plan on using 50 uL/tube to stain.

Thus, to start the dilution series, put 120 uL SM in an eppendorf tube, and add 2.4 uL of Ab (to make the 1:50 dilution). For the other dilutions (1:100 through 1:800), have 60 uL SM waiting in each tube. Vortex and "quick spin" the diluted Ab in a microfuge. Then take 60 uL of Ab dilution and add to the 60 uL SM in the next tube in the series (e.g., the 1:100). Repeat until you've made all dilutions. I make a little extra per tube to account for pipetting errors.

* Spin down tubes of cells at 1500 rpm x 5min. at 4 degrees C.

* Now suck off any supernatant (can use vacuum assembly with a Pasteur pipette with a yellow tip on the end). Rake each tube on the edge of your rack to start breaking up the cell pellet—this makes resuspension a little easier.

* Add 50 uL of SM to your "unstained cells", and then 50 uL of one antibody dilution to the remaining 5 tubes. This is your "staining" step. Flick, rake, or vortex to mix the cells and stain—make sure you don't have clumps.

* Put tubes back on ice in the dark for 30 minutes to stain. A Styrofoam box you can fit your rack in on ice and cover with a lid works well.

* When staining is done, add 3 mL of SM to each tube to wash.

* Spin at 1500 rpm x 5 min. x 4 C

* Aspirate supernatant.

* Rake tubes (without liquid) to break up cell pellets.

* Resuspend in 250 uL of diluted propidium iodide (PI) in SM. Use a 1 ug/mL working solution of PI. (Do a 1:1000 dilution of a 1 mg/mL stock.)

* ***Note: you should include whatever blocking or fixative steps you may have in your protocol when doing the titration. The PI is used to distinguish live from dead cells; you cannot use PI to determine viability when using a fixative.***

* Now analyze samples on a flow cytometer. Use the unstained sample to determine voltage (put cells in roughly the first log decade). Then read each of the dilutions without changing the voltage.

* Analyze data to determine which dilution is best for each antibody.

STAINING MEDIUM (SM):

1X HBSS (Hank's Balanced Salt Solution) **without** phenol red—very important
10 mM HEPES final conc. (do 1:100 dilution of 1 M, pH 7.2 stock solution)
2% FCS final conc. (10 mL FCS per 500 mL SM)