Titration of antibodies
Fluorescence Cytometry Core Facility

Why Titrate:

1.To save money.

Antibodies from most manufacturers are far in excess of what you need to stain all the molecules on cells. Generally you can effectively stain with a half to a tenth of what they recommend.

2. To optimize the separation of positive and negative populations, simplifying analysis.

If your antibody conjugate is bright and you have plenty of cells in the target population, this may not be as much of an issue. But for many surface and intracellular markers, the positive peak will not be clearly distinct from the negative peak. Titrating antibodies can significantly improve this.

Critical issues in titration:

1. Concentration of antibody is what counts; not staining volume per se or number of cells in that volume. This is related to antibody/antigen kinetics, since the goal in flow cytometry is saturation of the marker with the antibody. If you get your antibody concentration below saturation, you are not maximizing your staining, and that will show up in your titration as very dim staining at your low antibody concentrations. The purpose of titration is to find the optimum concentration where binding **saturation** occurs. As long as you are at that saturating concentration, cell number is no longer important. Bottom line: Always stain in the same staining volume, usually 50 - 100 µl, and add the amount of antibody that you optimize through titration for each antibody.

2. Pipetting very tiny volumes (1-2 µl) introduces error, but pipetting larger volumes from a diluted stock solution affects your staining volume. There are also some concerns about the stability of diluted antibodies, although if frozen or kept in Na Azide, Harlow says that diluted antibodies are stable for weeks to months. Bottom line: If you take titration seriously, you will probably find that you only need about a 10th of the amount of antibody recommended, and nobody can pipet less than a microliter effectively. Therefore, the best way is probably to do your titrations for the antibody, and come up with a stock dilution that will make your staining protocol as simple as possible and keeps your staining volumes fairly equivalent.

3. Staining conditions. Titration is time and temperature dependent. Titrate in the same conditions in which you plan to use the antibody.

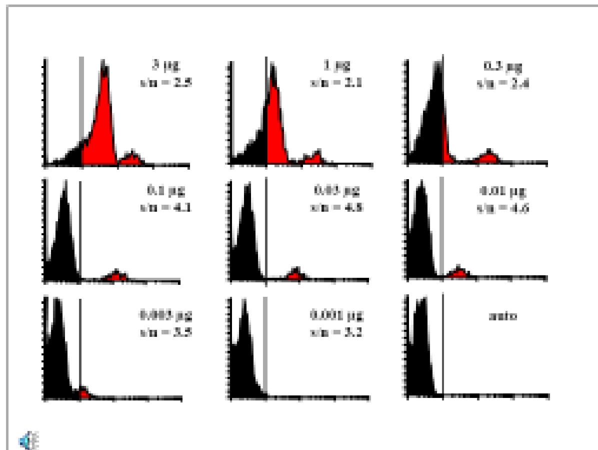
How to titrate:

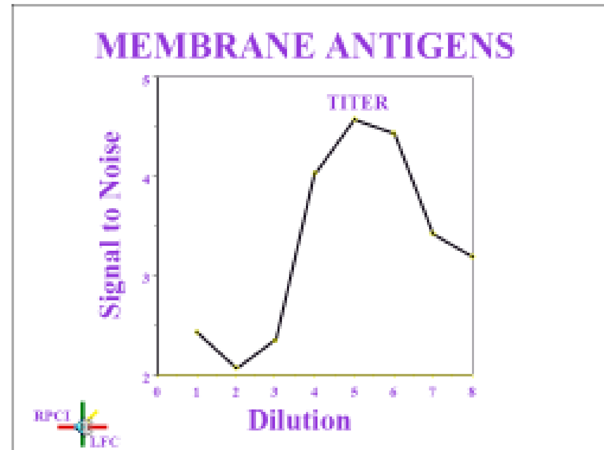
1. Easiest: Start at or slightly above recommended concentration, eg 10 µg/ml (1 µg/100 µl). So, set up 8 eppendorf tubes (or wells in a microtiter plate), with 100 µl of staining buffer in the first tube, and 50 µl in all the rest. Put 2 µg of antibody* into the first tube, which is usually about 1-2 µl from a commercial stock vial. Vortex. Transfer 50 µl from tube 1 to tube 2, vortex, and repeat for all tubes, discarding 50 µl from the last tube. Go back and add 50 µl of a cell suspension to all of the tubes, so all are at 100 µl. Stain as you normally do, eg 30 min at 4°, in the dark.
Run the washed cells on the flow cytometer, collecting data for all of the tubes.

For Homogeneous populations (clear pos/neg peaks) calculate the ratio of the MFI of your positive peak to the MFI of your negative peak for each (signal/noise ratio). You want the following results for optimum:

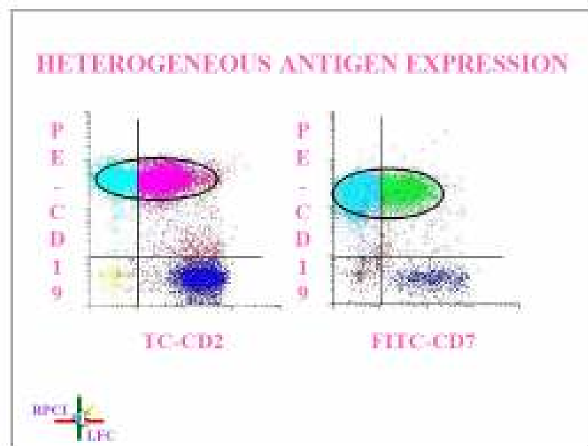
a) your negative peak is fairly low on the histogram, eg within the first quadrant.

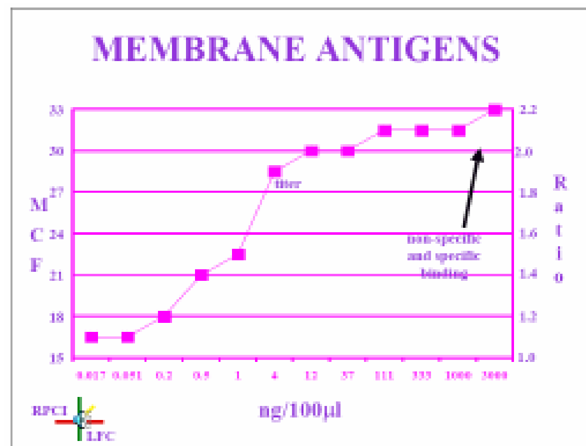
b) maximum signal/noise ratio.





For Heterogeneous populations (high variance in fluorescence intensity) graph the concentration vs. the signal and pick the point that just starts to level off.





2. Option: if you would like to find out the best dilution stock to keep for that antibody, you can set up a series of working stocks in eppendorf tubes (or wells) by adding 1-2 µl of your commercial stock to different volumes of storage/staining buffer, say 10, 20, 30, 40, 50 µl. Then set up your cell suspensions in staining buffer in separate tubes containing, for example, 90 µl of cells. Add 10 µl from each of the antibody dilution tubes to each of the cell suspensions, bringing each one to 100 µl total, vortex and stain. To convince yourself that cell number isn't critical, set up 2 sets of cell suspensions at different concentrations of cells, and stain with the same diluted stocks.

Perform the same analysis as above, and select the working dilution (and cell concentration?) that gives the best results.

Final Outcome: Calculate how many more samples you are going to be able to stain with that vial of antibody, and therefore how much money you are going to save!

Remember that it is concentration of the antibody that counts, so if you change staining volume, simply increase the amount of antibody to give you the same concentration. Cell number is not going to make as much difference, unless you are talking about 50 – 100 times more cells, like for a sort. Then you might want to add a little bit more antibody, maybe double your concentration.

* Remember that the concentration given on the vial (mg/ml or µg/ml) is for that antibody **and** any conjugate stuck to them! The mass of a purified antibody is NOT the same as an antibody conjugate, so 1 mg/ml of purified antibody is a WHOLE LOT more antibody than 1 mg/ml of conjugated antibody. You will need to titrate antibodies separately, depending on whether they are conjugated and which conjugate they have.

References:

Harlow, E., and D. Lane. Using Antibodies: A Laboratory Manual. Cold Spring Harbor Press,

Purdue web site: http://www.cyto.purdue.edu/hmarchiv/cytomail.htm

Dartmouth web site: http://www.dartmouth.edu/~celllab/flow/staincrit.html

DVD, Lecture notes from 29th Annual Course in Flow Cytometry, June 2006