# Titration of antibodies

**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 or more 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, 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 $10^{th}$ 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.

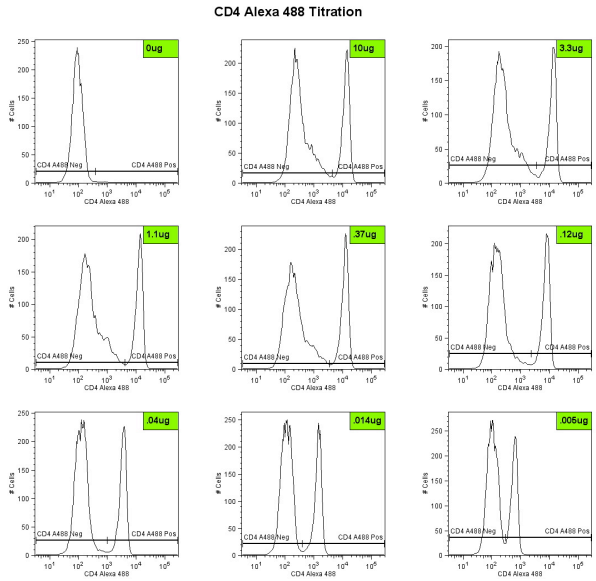
**How to titrate:**

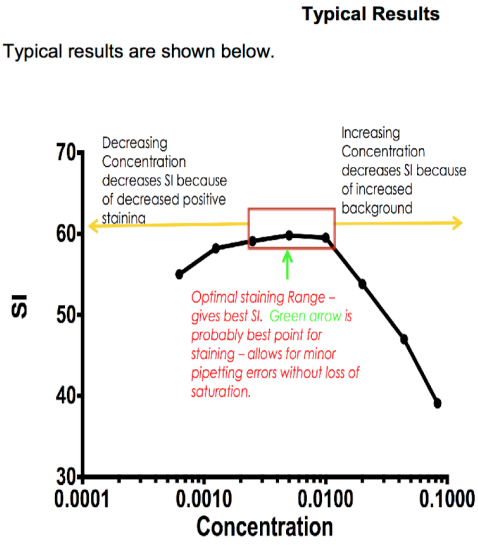
Start at or slightly above recommended concentration. Set up 8 eppendorf tubes (or wells in a microtiter plate), with 100 µl of staining buffer in the first tube, and 50 µl in all the rest. Put recommended amount of antibody into the first tube, which is usually about 1-2 µl from a commercial stock vial (you will need to calculate this based on concentration given and recommended amount per test). 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, e.g. 30 min at $4^{o}$, in the dark. Run the washed cells on the flow cytometer, collecting data for all of the tubes, using the recommended optimal PMT for the detector. Only lower the voltage if the positive signal is off scale. Depending on what cells you are using, you may need to add another marker/gate to find your cells of interest (e.g. CD45 to find WBCs in blood.)

For populations that show a clear pos or neg peak on a histogram or a dot plot, put a marker around the positive signal and another around the negative signal. For each dilution you will need to adjust the gates. Document the Mean or Median Fluorescence Intensity (MFI) of both the pos and neg populations and the Standard Deviation (SD) of the neg population. Calculate the Staining Index (SI).

$$SI = MFI\ pos - MFI\ neg/2xSD\ neg$$

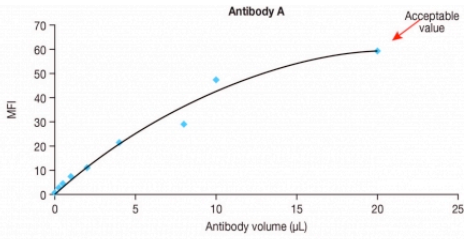
You will want to use the dilution that gives you the highest SI or Signal to Noise ratio.



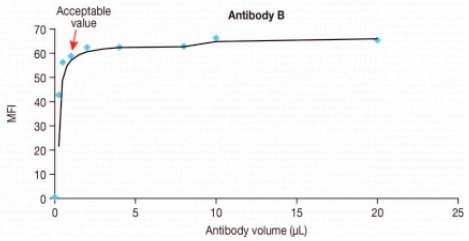




For populations that show up or down regulation of expression graph the concentration vs. the signal and pick the point that just starts to level off. In some cases, the recommended amount may be correct as in the first example here, or may plateau wasting valuable antibody and producing more non-specific binding and background noise.

| Antibody A | |
|---|---|
| Antibody volume (µL) | MFI |
| 0 | 0.62 |
| 0.25 | 2.73 |
| 0.5 | 4.41 |
| 1 | 7.35 |
| 2 | 11.00 |
| 4 | 21.39 |
| 8 | 28.96 |
| 10 | 47.31 |
| 20 | 59.23 |




| Antibody B | |
|---|---|
| Antibody volume (µL) | MFI |
| 0 | 0.45 |
| 0.25 | 42.73 |
| 0.5 | 56.16 |
| 1 | 58.86 |
| 2 | 62.35 |
| 4 | 62.45 |
| 8 | 62.75 |
| 10 | 66.22 |
| 20 | 65.34 |




**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!

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