Notes on titering antibodies

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Why titer antibodies?

To optimize signal, achieve saturation, minimize background, and conserve reagents

Literature and web resources on titrations

The theoretical aspects of titration of antibodies is covered in Kantor, A & Roederer, M. "FACS Analysis of Leukocytes" in **The Experimental Handbook of Immunology, 5'th edition** . Lenore Herzenberg, Leonard Herzenberg, Caroline Blackwell, Donald M. Weir, eds. More can be found on the Herzenberg lab website.

Reporting concentrations

Absolute vs. relative concentrations

There are two basic ways of reporting antibody concentrations: absolute and relative. Each has its place.

- **Absolute concentrations** are usually reported in units of mass/volume, such as mg/ml, µg/ml, or ng/ml. Sometimes, they are reported in odd units such as µg/100 µl (useful if your standard staining volume is 100 µl, but otherwise inviting confusion), but these can always be converted to more regular concentrations. Of course, antibody concentrations can also be reported in molar-based units such as µmol/liter (µM) (such units are especially useful during the preparation of dye conjugates, for the calculation of molar ratios of dye to antibody). Absolute concentrations should have meaning from lot-to-lot of master stocks with different concentrations.
- **Relative concentrations** are reported in units such as 100x (for master stocks) or dilutions ***relative to a master stock***, such as 1:1000 (often presented in figures as the reciprocal). Relative concentrations are often less cumbersome in the lab, but they have no meaning if the concentration of the master stocks vary from lot-to-lot.

The concentration of antibody stocks, particularly from commercial suppliers, are sometimes reported in units such as µl/test. This unit only has meaning with reference to a standard test volume. Often, this is assumed to be 100 µl, so if you are using 200 µl staining reactions, you should consider doubling the volume of antibody added to your stain.

When you are reporting concentrations, you must always include data which permits the calculation of the absolute concentration, even if you are generally using relative concentrations. For example, it's perfectly valid to report a working stock as 20x, as long as you also report that it's at 400 µg/ml (if that's what it is). For a dilution series, this means that you only need to report the absolute concentration for the highest tested concentration.

Titering Methods

There are a variety of methods for titering antibodies. If performed with sufficient care, all should give the same results. Among those methods are:

1. Construct a serial dilution series of working stocks (e.g. in eppendorf tubes, or in 96 well, round bottom plates), and deliver a constant volume (e.g. 5 or 10 µl) from each member of the working stock series to the corresponding staining reaction (total volume, 100 µl). **This is my preferred method.** Remember, when reporting titers, report the final titer, not the dilution of the intermediate working stocks.
2. Prepare a single working stock (e.g. at an antibody concentration of 50-100 µg/ml), and add decreasing amounts to each of a series of staining reactions. Without preparing additional dilutions of the working stock, this technique can only cover a 20-fold range (e.g. 20, 10, 5, 2.5, 1.25 µl of the working dilution to the respective stains); if you need to cover a greater range, you'll have to perpare one or more intermediate working stocks (but not as many as above). If you want to get picky, you could add an appropriate volume of FACS buffer to each tube to keep the total volumes of all stains the same, but omitting this will probably lead to a negligible error.
3. Serial dilutions of the antibody to be titered can be prepared in the same tubes used for staining, as long as the dilution series is the first thing that is added to each tube. For example, you could do the following: (a) add 10 µl of FACS buffer to tubes 2-12 in a series, (b) add 20 µl of the working stock to tube 1 in the series; and (c) withdraw 10 µl from tube one, add it to tube 2 and mix, and then repeat this procedure in subsequent tubes. This can get a bit tricky because it's difficult to handle small volumes (e.g. 10 µl) in 12 x 75 FACS tubes.
4. In a variation of method 3, you could prepare a serial dilution series in which the volume of each series member, before the addition of the cells, is 50 µl. Then, you add 50 µl of a 2x concentration of cells (e.g. 1E7 cells/ml in order to add 500,000 cells total) to each stain. Of course, when reporting the stain concentrations, you should factor in the 2-fold dilution that occurs upon addition of the cells. This method works extremely well for PBMC (and mouse splenocytes), but I'm not sure if there will be complications if it is used for human whole blood samples; it's unlikely, but there might be problems with red cell lysis if the blood is diluted 2-fold through addition of the stains.

The bottom line? I like method 1, but your mileage may vary.

Master stocks, working stocks, and staining reactions

There are three relevant concentrations in an antibody titration experiment:

1. The concentration of the master stock (typically 0.2-4 mg/ml).
2. The concentration of the intermediate "working" stocks.
3. The concentration in the staining reaction itself.

When you are reporting the concentrations in a titration experiment, always report the concentration in the staining reaction, not the intermediate working stocks.

For example, if you prepare a 1:100 dilution of a master stock and use 5 µl per 100 µl of blood, the reported dilution is 1:2000, and NOT 1:100. Of course, as noted above, it is also necessary to somewhere report the absolute concentration.

Typical antibody concentrations

When performing an antibody titration experiment, the highest concentration staining reaction should contain at least 20 µg/ml of antibody (corresponding to 2 µg/100 µl). This means that if you are adding 5 µl of an intermediate working stock to 100 µl of cells (or whole blood), then the concentration of the working stock should be 400 µg/ml; alternatively, you can add 10 µl of a 200 µg/ml working stock to 100 µl of cells. The idea is to start with an antibody concentration that ought to be at least 1-2 dilutions above the saturation level.

You should perform 8-12 2-fold serial dilutions from the highest concentration of your intermediate working stock. You should perform enough serial dilutions so that you achieve concentrations that are clearly subsaturation. The table below is included as an example of how to set up a titration series. If the concentration of the master stock is 4 mg/ml, then you should prepare an intermediate working stock that is 400 µg/ml (a 1:10 dilution), and the prepare a serial dilution series or working stocks from that working stock. Choose a volume for the working stock that you can pipette accurately and precisely, but that does not waste reagents. For example, your first working stock could be prepared by pipetting 5 µl of the master stock into 45 µl of FACS buffer. Subsequent dilutions could be prepared by pipetting 20 µl from this working stock into 20 µl of FACS buffer, and so on in a series. Then, 5 µl from each of these working stock dilution series members could be added to each staining reaction.

Sample titration experiment			
Test#	Dilution of Working stock (relative to master stock 4 mg/ml)	Final dilution (relative to master stock at 4 mg/ml)	µg/ml in the staining reaction
1	10	200	20
2	20	400	10
3	40	800	5
4	80	1600	2.5
5	160	3200	1.25
6	320	6400	0.625
7	640	12800	0.3125
8	1280	25600	0.15625

Use counterstains when appropriate, as in the following examples

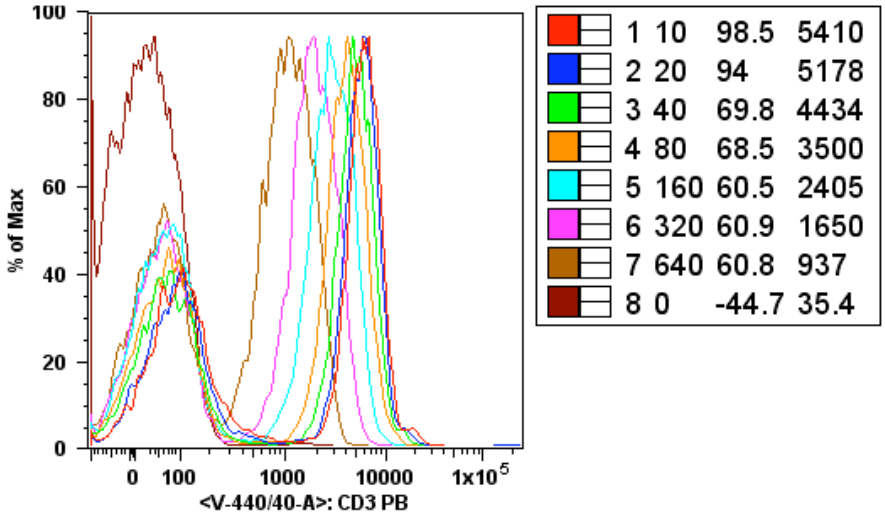
When titering CD8 mAbs, stain each sample with constant amount of a CD3 antibody, usually labeled with a fluorophore that doesn't interact with the label on the antibody you are titering.

When titering an anti-HLA-DR mAb, you might use as counterstains three antibodies: anti-CD3, anti-CD8, and a B cell marker such as CD19. For some anti-HLA-DR mAbs, the staining intensity on B cells is so bright that the signals are difficult to compensate, and it is necessary to use more dilute, subsaturating concentrations.

When titering CD3 mAbs, it's not clear what you should use as a counter stain. Perhaps you can omit it.

Data analysis

Titration data are usually plotted as histograms (see below), but they could also be plotted as dot plots. Using the FlowJo Layout Editor, you can easily prepare histogram overlays that give you a nice, compact summary of the data. Alternatively, you can batch a series of histograms and present them in a vertical orientation (so that you can more easily compare shifts in the peak position), but given the availability of overlays, I don't see much point in this. FlowJo version 6.0 has added useful features and flexibility in Layout legends that make this an even more attractive option. For example, for every sample, you can add the antibody concentration as a new keyword, and insert it into the legend. You can also add statisitics to the legend, such as the median intensities of the positive and negative populations. These are very nice new additions, and I recommend that you explore them.



In addition to plotting the data, you should use FlowJo to calculate the median of both the positive and negative populations. For a titration series, this means that it is likely that you will have to adjust the applied gates for each sample, but this can be done very quickly. As noted above, you can add these statistics to the figure legend.

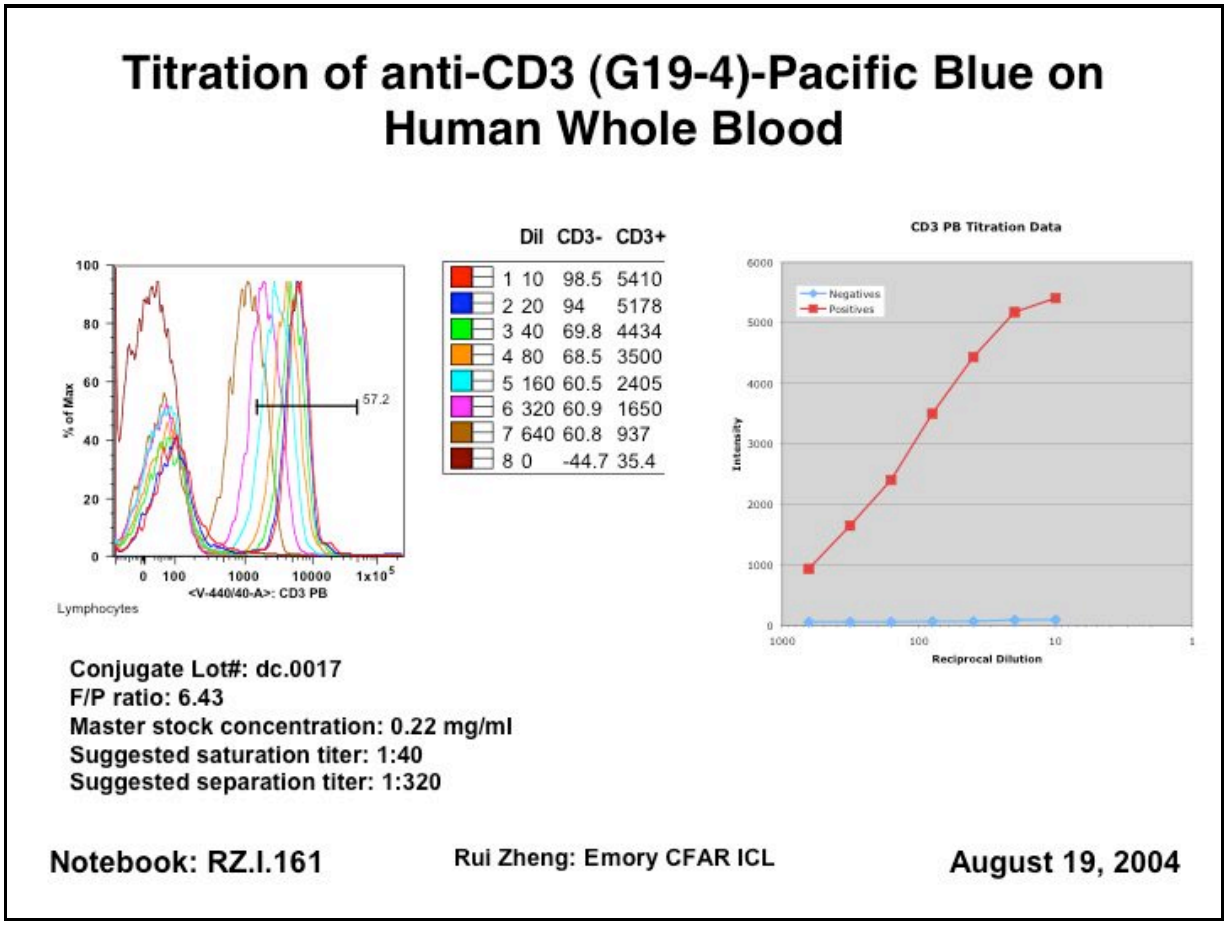
The Herzenberg lab website presents an interesting alternative method for calculating the intensities of the median of the positive and negative populations, using the concept of percentiles. This method is well worth a look.

Finally, you should plot both the median intensities of both the positive and negative populations, using a program such as Excel or Kaleidagraph. Plot separate curves for positive and negative populations. See the example slide below.

Organizing your analyzed data

It is helpful to maintain either a database or a "portfolio", perhaps in PowerPoint, of your data, so that it can be perused at a quick glance. One recommended slide layout is shown below. The important elements of the data are:

1. Overlaid histograms of the raw FACS data.
2. A legend of the FACS data, augmented to include columns for the reciprocal dilution (Dil), and the median fluorescence intensities of the positive and negative populations. The methods for modifiying the overlay legends are described at the FlowJo website.
3. A scatter plot, prepared in Excel, of the reagent dilution versus the fluorescence intensities, for both the positve and negative populations.
4. A text box, containing the following: (a) Conjugate Lot#, (b) F/P ratio of the conjugate, (c) the concentration of the master stock in mg/ml, (d) the suggested titers at saturation and separation
5. Separate text boxes containing a cross-reference to a notebook page ("RZ.I.161"), the date of the experiment.
6. A footer on the master slide, containing the investigator name and lab.



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