Institut für HIV Forschung
SOP #06-03 (Nov 2017 - BS)

Antibody Titrations

Reagents

Reagent	Vendor	Catalogue #	Stock Conc.
FACS Tubes	BD Falcon	352054	-
Anti-CD28/CD49d Antibody	BD Fastimmune	347690	-
GolgiPlug (Brefeldin A (BFA))	BD	555029	-
GolgiStop (Monensin)	BD	554724	-
Cytofix/Cytoperm Kit	BD	554722	-
Live/Dead Fixable Aqua Dead Cell Stain Kit	Biolegend	423102	-
SEB	Sigma	S4881	1mg/mL
PHA	Fisher	R 30852801	250ug/mL
PMA	Sigma	1585	1mg/mL
Ionomycin	Sigma	141128	1mg/mL

This protocol is for titrating individuals antibodies to give the best resolution staining for an experiment. Many conjugated antibodies come with a recommended staining concentration, and while this is a good starting point, this suggested concentration was not determined using the same experimental conditions that will be used in your assay. Therefore, it is important for each antibody that will be used in the assay to empirically determine which concentration works best.

Background

Titrating antibodies can improve your flow data analysis in several ways. Cells that are understained will not have sufficient separation between the positive population and negative population (see Figure 1; red is positive population while blue is negative). Cells that are overstained will have high background, in that the negative population will be shifted towards a higher fluorescence. Therefore, it is advised that a new titration be performed whenever a new antibody is used or when new experimental conditions are introduced.

It is also important to note that while titrations help in flow analysis, they also are often a cost saving technique. Typically, antibodies can be used at lower concentrations than the manufacturer recommends, and even using 1-2ul less antibody per test than the recommended amount will save a lot of money over several experiments. Some antibodies can continue to produce data even when stained at concentrations that are 64-fold more dilute than recommended. Thus, antibody titrations should be tested using several dilutions below the manufacturer's suggestion.

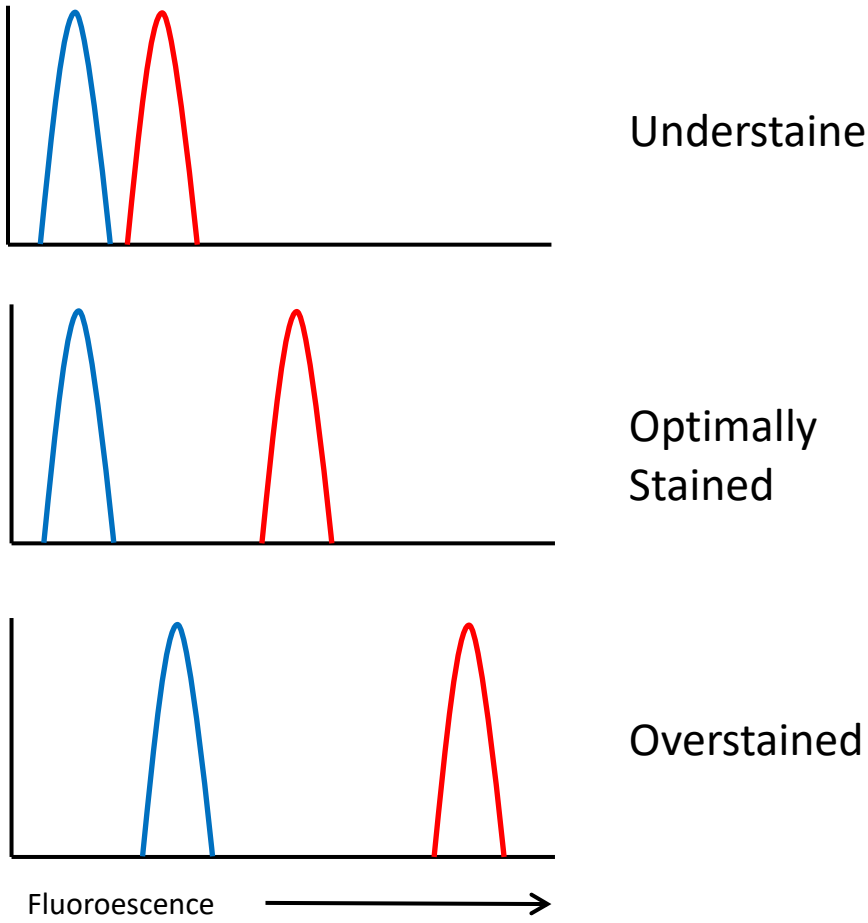

Figure 1 – Graphical representation of different staining possibilities

Titrations are dependent on several variables including time of staining, temperature, and condition. Hence antibodies titrated for surface staining need to be titrated again if used in an ICS protocol. It is also important to note that there are two different concentrations that one might use: the "saturating" concentration and the "separation" concentration. The saturating concentration is the concentration of antibody that gives the maximum fluorescence for that antibody, while the separation concentration is the concentration which gives the best separation between positive and negative populations. Deciding what is the "best separation" is a rather subjective, and while the separation concentration is typically less than the saturating concentration, it can still have close to maximum fluorescence, though it may be more sensitive to changes in staining conditions. Thus, it is very important to titrate your antibodies in the **exact same conditions** that you plan to use them.

Finally, if you plan to stain 10x more cells, then this does NOT mean to use 10x more antibody. The concentration used to stain 50,000 cells can also be used to stain 1,000,000 or 10,000,000 cells. When planning to stain significantly more cells, another quick titration can be done, but typically one will only need 2-5x higher concentration at most. With all that in mind, use the following protocol to titrate your cells:

Staining Protocol:

1. Titrations should be performed using only the antibody of interest.
2. Prepare your samples as you normally would for your assay (SOP #06-00). You will need 9 samples per antibody being titrated, plus an Unstained control. Label the tubes 1-9.
    a. When titrating certain surface antibodies and any ICS antibodies, be sure to use a stimulated (using a superantigen) and unstimulated condition for each dilution being tested. This is to ensure proper gating during analysis (so in this case instead of having 9 tubes, you would have 18)
3. When you arrive at the staining step for the antibody being titrated (either surface or ICS), decant/aspirate all of your samples. Vortex briefly.
4. For the titration, we will create nine 2-fold dilutions. Place nine 1.7ml Eppy tubes in a rack. Add 270ul of the same buffer that your cells are resuspended in to the first Eppy tube and 150ul to the remaining 8 Eppy tubes
5. Since the manufacturer *usually* recommends the saturating concentration, we will begin at 2x this concentration and work our way down. Pipette 2 times the recommended concentration of antibody into Eppy tube "1" (e.g. if the suggested dilution for an antibody is 1:20 per test and we want a 2x dilution for the first tube which is 1:10 then add 30ul of antibody)
6. Pipette mix the first tube thoroughly.
7. Aspirate 150ul of the antibody mix in the first tube and transfer to the second tube, taking care not to touch the tip to the solution.
8. Discard the tip and get a new one. Pipette mix the new dilution thoroughly.
9. Repeat Step 7&8 for tubes 3-9.
10. Transfer 100ul the antibody mix in Eppy tube "1" to the FACS tube labeled "1". Transfer 100ul of antibody mix from tubes 2-9 to their respective FACS tubes. Your FACS tubes will have these dilutions now:

Tube #	1	2	3	4	5	6	7	8	9
Concentration (times recommended)	2x	1x	1/2 x	1/4 x	1/8 x	1/16 x	1/32 x	1/64 x	1/128 x

11. Briefly vortex your samples and incubate at the temperature and time your protocol dictates
12. Wash, resuspend, and perform FACS analysis as you normally would.

Analysis Protocol:

When analyzing your titration data, it is important to get as close to the population of interest as you can. The gating strategy shown below can be used for a majority of titrations performed here.

The following example is for a CD3 antibody:

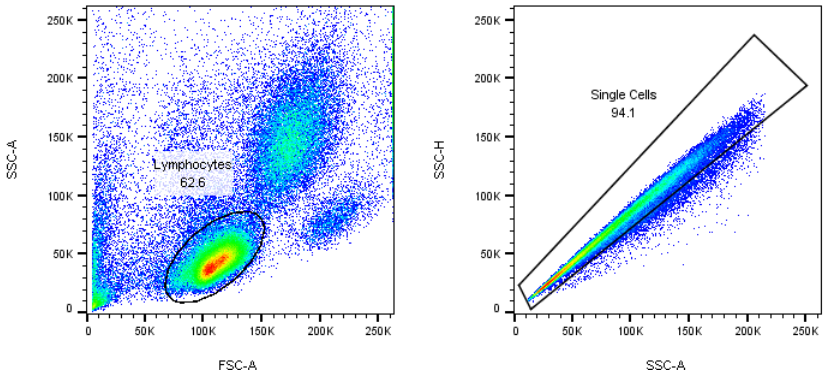

Figure 1 – Example gating strategy

In Figure 1, we first gate on the target population (lymphocytes) then in the graph on the right, we are gating on single cells (AKA singlets). The two gates can be drawn in either order, but be sure to research the marker you are titrating and on what cells it is expressed! If the marker is expressed primarily on innate cells (i.e. monocytes, macrophages, DCs), then they tend to be a bit bigger/more granular. Those are located in the cell population above and to the right of the lymphocyte population.

Now it is time to view your marker. Click on an axis title and choose the fluorochrome/marker that you are analyzing. On the other graph you can choose either FSC-A (Figure 2, left) or view it as a histogram (Figure 2, right).

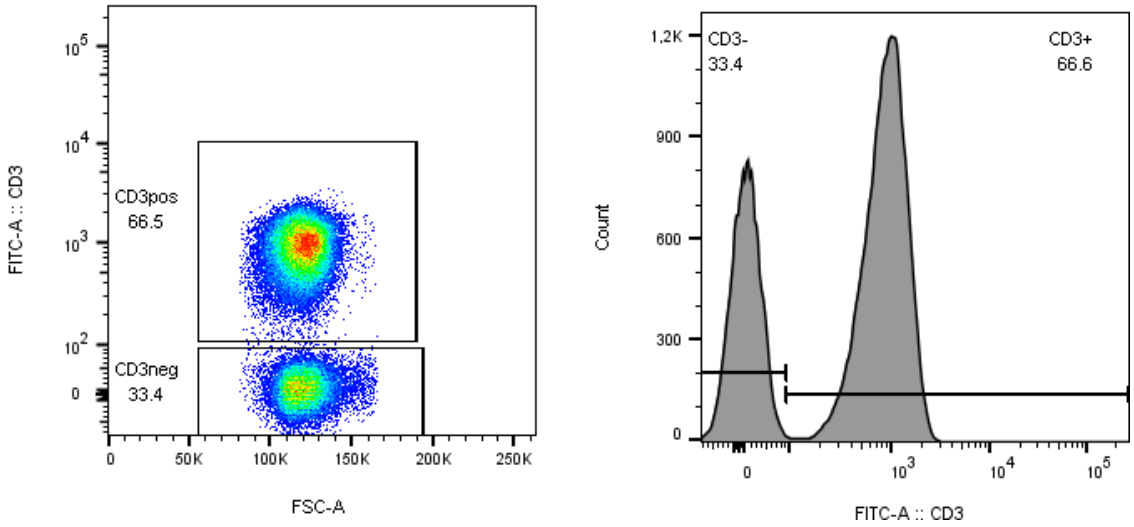

Figure 2 – Comparison of gating with FSC/pseudocolor graphs and historgrams

With titrations, it is important to have a population that contains cells that are negative for the marker you are titrating. Sometimes, a negative population does not lie within your first gate, so it may become necessary to gate on all cells like so:

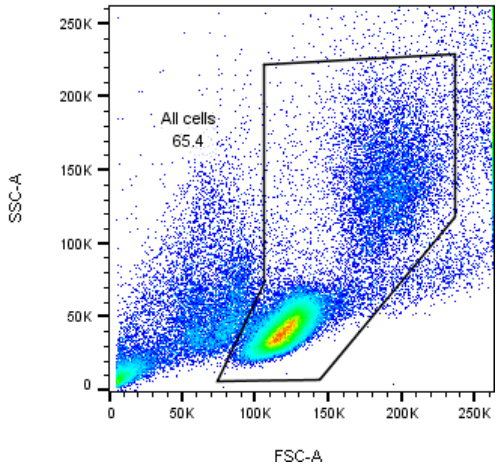

Figure 3 – Example of expanding initial gate

After you have a positive and a negative population in view, you can begin drawing your gates around each. As you go through your dilutions, you should see your positive population move towards the negative. It is very important that the gates for each sample are adjusted so that they completely surround their corresponding population. The same gate cannot necessarily be copied to every sample as this will give inaccurate results later on. Figure 4 shows how gates are adjusted between dilutions of the same marker.

In some instances, your positive and negative populations will begin merging. When this happens, use the gates from the lowest dilution where the two populations are still separate, and copy it to the remaining dilutions.

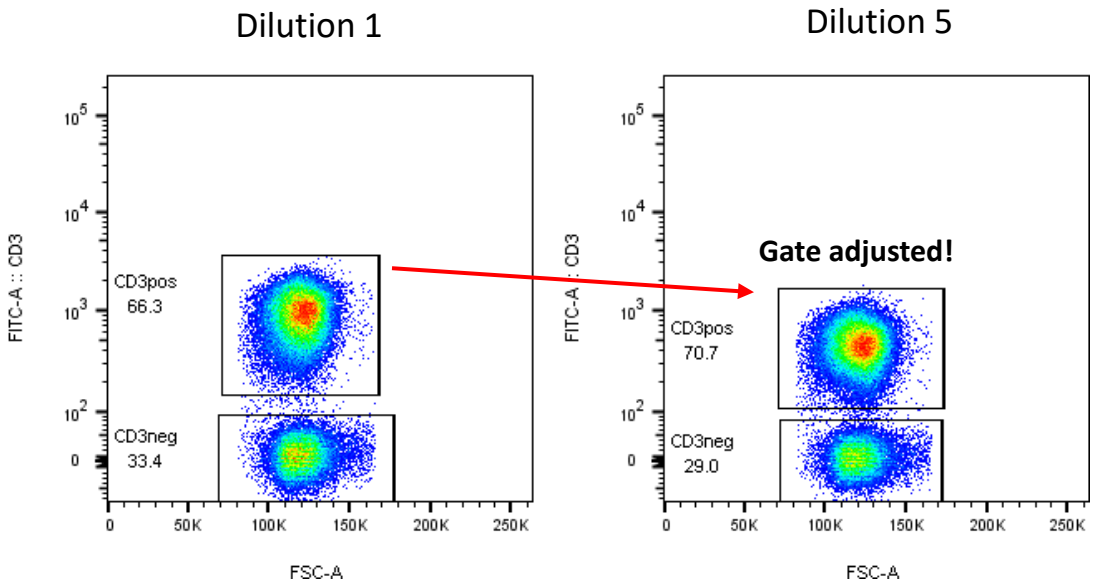

Figure 4 – Example of gate adjusting between dilutions in FlowJo

The gates in Figure 4 were drawn using pseudocolor graphs, gates can also be drawn using histograms or for difficult populations the contour plot can also be used.

After all the gates are drawn for both your positive and negative populations, it is time to compile the median fluorescence intensities (MFIs) of the marker you are titrating.

1. On the main window in FlowJo, right click on the positive gate (in the examples above, I would click on "CD3pos") and choose "Add Statistic…"
2. In the left column of the window, click median, then under "Parameter" choose the correct fluorochrome/channel for the antibody you are titrating. (In the example in Figure 4, CD3 Alexa Fluor 488 was being titrated, so I would choose "FITC-A" since Alex Fluor 488 is measured in the FITC channel).
3. Finally, click "Add" at the bottom and you should now see an "Σ" symbol under your positive population in the main window. Copy this statistic to all positive and negative populations (for all dilutions).
4. Under your negative population only, go to the "Add Statistic…" menu as done in Step 1.
5. Following the same procedure in Step 2, add the "Robust SD" statistic to your workspace
6. To compile your MFIs and robust standard deviation (SD) into one table, click the "FlowJo" tab in the main window, click "Table Editor" and a new window should appear. Drag the statistics (for example click and drag the "Σ Median…" line) from **one** positive population and **one** negative population to the open white space in the Table Editor window. The robust SD should only come from the negative population. You should now have 3 lines in the Table Editor window, 2 for the medians and 1 for the SD. Click "Create Table" and copy the contents of the new table to Excel.

Next we will calculate the Stain Index (SI) for each dilution of the titrated antibody where SI is:

$$SI = \frac{MFI_{pos} - MFI_{neg}}{2\sigma_{neg}}$$

Where σ_{neg} is the robust standard deviation of the negative population.

Using this formula allows us to choose which dilution yields the brightest, most easily discernable results. If we were to only assume that the dilution which reaches the saturation point (highest MFI) was best, we may have incredibly high background. Below is an example of a CD8 antibody titration:

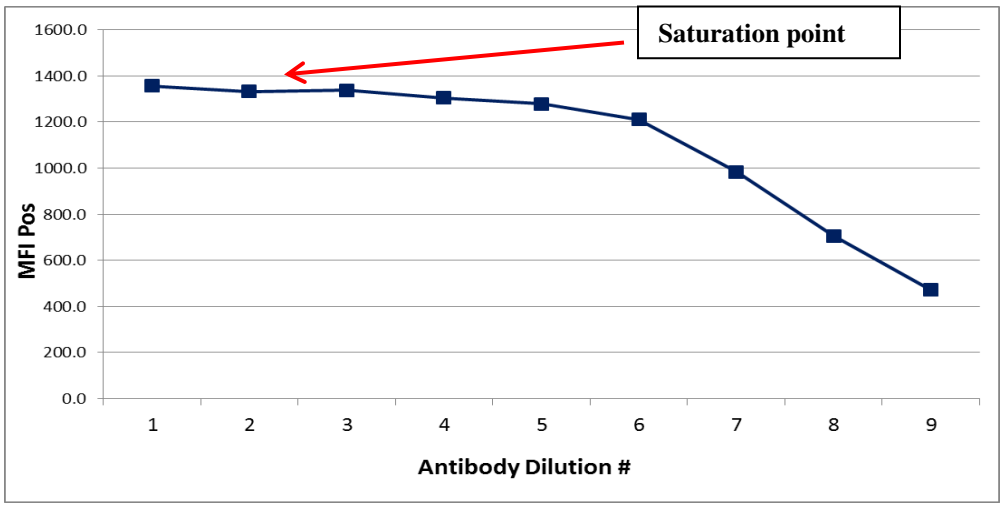

Figure 5 – Graph of MFIpos vs Antibody Dilution

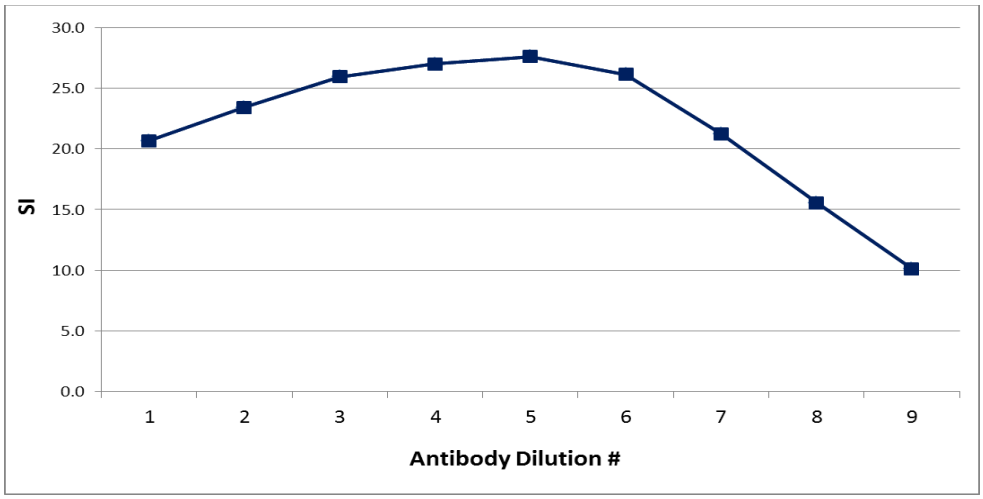

Figure 6 – Graph of SI vs Antibody Dilution

For both Figure 5 & 6, the x-axis displays the dilution number which corresponds to the concentration of the antibody (see table on page 3 of this SOP). However, in Figure 5 the y-axis shows the MFI_{pos} while in Figure 6 we see the SI on the y-axis. If we were to choose our optimal dilution based on Figure 5 (that is the MFI_{pos}), then we would pick the highest dilution number that is close to the saturation point so in this case it would be either #4 or #5. But, if we look at the actual flow histograms we can see another factor that would influence our ability to read the results, the spread of the populations.

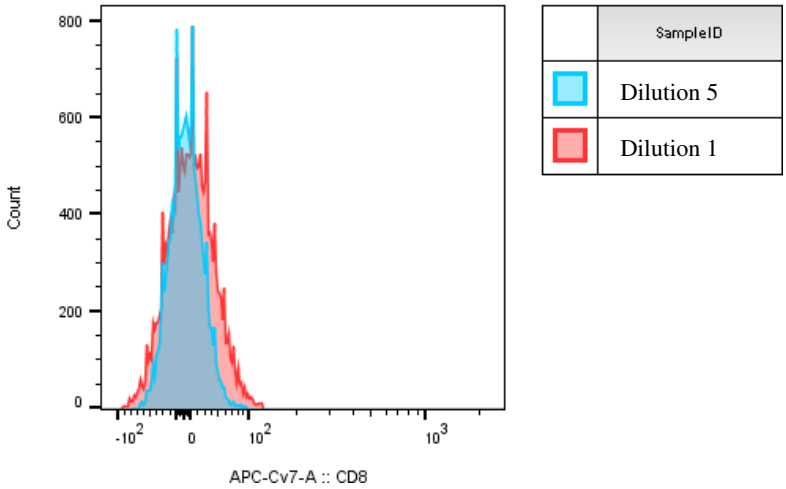

Figure 7 – Comparison of negative populations between dilutions

In Figure 7, we can see that Dilution 1 has a larger spread in the negative population, which tells us that it is slightly overstained. Dilution 5 has a lower spread and would have even better separation between itself and the positive population. This is why we use the SI to determine the optimal dilution as it incorporates the SD into the calculation and thus takes into account the spread of the negative population.

Choosing the optimal dilution requires one to consider several factors such as highest SI, which dilution has a MFI_{pos} close to the saturation point, and where the negative population lies. Calculating SI is a great way to quickly get an idea of which dilution would be best used, but one should always go back to the

actual flow plots and use good judgement when making a final decision. In our case in Figure 5-7, dilution 5 would be the best choice.

SI Calculation Protocol

1. Open the Antibody Titration Template (Abteilungsverzeichnis → Flow Resources → Antibody Titration Template)
2. Fill in the information at the top of the sheet (Staining method, antibody, etc.)
3. Copy your sample names to the first column of the sheet (be sure the Ab vol and dilution column match your order, if not then adjust the values as needed)
4. Copy the positive MFI values for your antibody to the "MFI POS" column
5. Do the same for the negative MFI values to the "MFI NEG" column and the robust SD to the "SD NEG" column
6. Determine which dilution has highest SI
7. Double check the flow plots in FlowJo and decide whether your calculations agree with what you see visually
8. Record in the excel file the optimal antibody volume and dilution ratio that will be used for future experiments in the highlighted (yellow) boxes.
9. Save the excel file in the Antibody Titrations folder located in the same location you found the template.
    - File name = "[Manufacturer] [Antibody] [Fluorochrome] [Clone] [Surface/ICS]"
10. Update the lab Antibody List with the optimal dilution and your initials
11. Copy your FCS files and workspace to the Abteilungsverzeichnis/ Flow Resources/FCS Files folder so that others may see the gating in the future if needed