Titrating Antibodies for Flow Cytometry

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Why Titrate?

Antibody titration is one component of the larger process of panel optimization. Titration is very important for optimizing resolution and obtaining robust results for population identification and expression level measurements. It takes a little time and effort, but it can save time, money, and angst in the long run.

Save money

It is often possible to use less antibody than recommended by the supplier. This means you save antibody and therefore money on your experiments.

Improve accuracy of staining and decrease non-specific binding

Titration allows you to determine the amount of antibody that gives you the best separation of populations in your samples, and the best measure of expression levels. Too little antibody means the cells expressing the marker of interest do not stain as brightly as they could, and may not separate adequately from the negative cells. Too much antibody can increase non-specific binding, which increases the spread and background of the negative population. Both situations result in lower resolution of the measurement.

The Wet Work

It is best to titrate antibodies under the same staining conditions you will use in your experiment. During the titration, however, each tube will contain only one antibody.

Test a range of antibody amounts above and below the amount recommended by the supplier. For example, for an antibody with a suggested volume of 5 µL per test, you might try 0.5, 1, 2.5, 5, and 7.5 µL. Include a sample with no antibody as well.

When acquiring data on the flow cytometer, be sure that the stained cells are on scale; look at the tube with the highest concentration of antibody for each titration before recording any samples. Aim for 20,000 live, single cells in each data file. You might need more events if you are looking for a very rare population. It is important to have sufficient numbers of both positive and negative events. This can be difficult when staining homogenous cell lines.

With only one antibody per tube, compensation is not necessary.

References

http://expertcytometry.com/what-is-titratio/

http://ucflow.blogspot.com/2009/06/antibody-titrations.html

http://docs.flowjo.com/d2/workspaces-and-samples/samples-and-file-types/ws-export/

Staining Tips

Use the same cell type(s) you will use in your real experiment.

Include a viability marker so dead/dying cells don't skew the results, as dead cells tend to stain non-specifically.

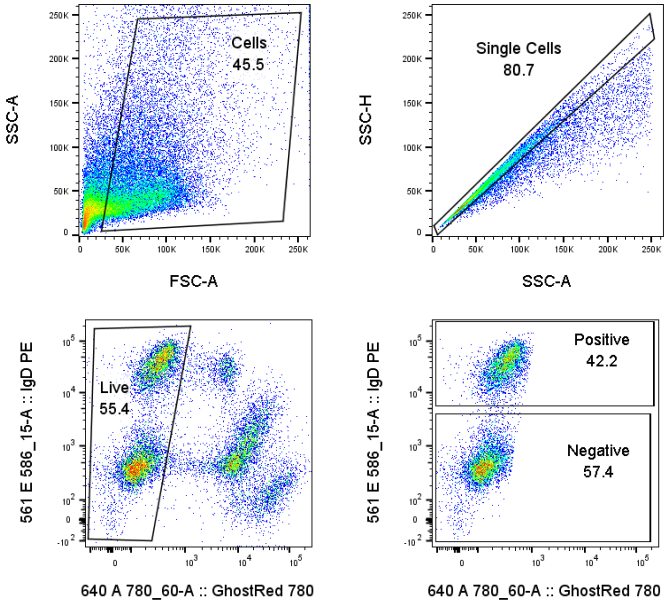
While varying the concentration of antibody per reaction, keep the total staining volume the same for all tubes.

Use the same number of cells for each tube.

Dilute the antibody stock rather than trying to pipet very small volumes.

Analysis

Begin by gating for live, single cells, and drawing broad gates to define the positive and negative populations. It may be necessary to adjust the positive and negative gates individually for each sample if there are large shifts in the populations.



Calculate the **Separation Index** (SI) for each sample. This metric is similar to the Staining Index, but the denominator has been modified to weigh the negatives on the right side of the distribution. Each term in the equation refers to a statistic derived from the fluorescence intensity of the antibody staining. The higher the Separation Index value, the better the separation between positive and negative populations.

$$Separation\ Index = \frac{MedianPositive - MedianNegative}{(84\%Negative - MedianNegative)/0.995}$$

Concatenating all the live, single cells from each tube of the titration can help with visualizing the data, as shown in the plot to the right.

Instructions for calculating SI and making concatenated files in FlowJo can be found online or in other UWCCC Flow Lab tech notes.

In this example, all concentrations tested show separation between the positive and negative populations. It is best to aim for maximum separation, because spectral overlap from other colors may reduce resolution in the context of the full panel. The sample stained with 2.5 µL of antibody produced the best separation, as evidenced by the Separation Index. The visual display shows that increasing the amount of antibody increases staining of the negative population without improving staining of the positive population.

Tips for Annotating Data

Minimize typing in DIVA by naming the Specimen for the antibody and the Tubes for the amount/concentration. Duplicate and rename the Specimen without renaming each Tube.

Organize samples in FlowJo by making a Group for each antibody. Include the no-antibody (viability only) control in all groups.