Titration of Antibodies for Flow Cytometry

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Purpose:

To determine the optimal (saturating) antibody concentration which produces the highest signal without increasing background.

Supersaturating concentrations will increase background and non-specific binding and is not cost-effective. Non-saturating concentration may cause sample-to-sample variation and decrease resolution and sensitivity. At saturating staining concentrations the amount of antibody present is not limiting and is sufficient to stain all relevant antigens without significantly lowering the concentration of free antibody (see *Kantor, A. and Roederer, M. (1997)* for examples of this calculation). Therefore the antibody concentration but not the number of cells is critical for optimal staining.

The optimal antibody concentration must be determined for each application and set of experimental conditions (including staining time and temperature) and is determined by using a series of dilutions. The manufacturer's recommended amount should only be used as a reference "starting point" since their titration conditions may not be identical.

Materials:

Staining media (SM)
Sterile filtered calf serum (CS)
0.1% trypan blue
1 mg/ml propidium iodide (PI) [in dIH_2O]
Nitex mesh, 85-µm mesh size
4-ml conical tubes [Diamed STK 8550]
5-ml round bottom tubes [Falcon 2052]
Serial dilution of antibody in SM
Cells appropriate for antibody

Protocol:

1. Select appropriate tissue from which to make a single-cell suspension for staining. When titrating antibodies, it is important to have both positive (stained) and negative (unstained) cells within the population to allow a calculation of signal-to-noise ratios. Isolate enough cells for 1-2x10^6 cells/tube. Remember to include a tube for unstained cells. For titration of 2º antibodies or 2nd stage reagents include a 2º alone control tube.

2. Prepare 2-3-fold serial dilutions of antibody in SM (*eg.* 50 µl antibody + 100 µl SM for 1:3, then 50 µl 1:3 dilution + 100 µl SM for 1:9 dilution, *etc.*). The starting concentration should be ~1X or 2X that recommended by the manufacturer or ~10 µg/ml purified IgG for most antibodies. Prepare at least

5 dilutions, more if you suspect that you have a really concentrated antibody preparation.

3. For titration of 2º antibodies or 2nd stage reagents, use a previously titrated 1º antibody at the standard dilution (2X saturating) and make serial 3-fold dilutions of the 2º antibody or 2nd stage reagent.

4. Prepare single-cells suspension of tissues.

5. Resuspend each cell pellet in 50-µl diluted antibody. Include a positive control for each antibody to be tested. This is usually the last lot of antibody used or, if using a new conjugate/antibody, an antibody with the same specificity, on the same cellular population. Incubate for 20-40 minutes on ice. Protect from light if fluorochrome-labeled 1º antibodies are being used.

6. Add 0.5 ml SM to each tube (*ie.* ≥10 staining volumes) and underlay with 0.3 ml CS using $5^3/_4$" Pasteur pipettes.

7. Collect cells by centrifugation at 400 x*g* for 5 minutes at 4ºC.

8. Aspirate supernatant. Resuspend cell pellet in 50 µl 2º antibody, if necessary. Protect from light. Incubate for 20-30 minutes on ice. If biotinylated 1º antibodies were used, incubate fluorochrome-conjugated avidin 2nd stage for 30 minutes on ice.

9. Repeat steps 6 and 7.

10. Aspirate supernatant. Resuspend cell pellet in 0.5 ml of SM containing 1 µg/ml PI. Filter through Nitex screens into labeled 5-ml round-bottom FACS tubes [Falcon 2052].

11. Collect data on flow cytometer.

12. Analyze data and calculate the stain index.

13. Select the dilution with the highest stain index.

Buffers:

Staining Media (SM)

1X HBSS with Ca^{2+}/Mg^{2+}; 2% calf serum; 10mM HEPES, pH 7.2
Aseptically add sterile calf serum and sterile 1 M HEPES, pH 7.2, to sterile 1X HBSS with Ca^{2+} and Mg^{2+}. Store at 4ºC.

0.1% Trypan Blue

Dilute 0.4% Trypan blue (Sigma, T-8154) 1:4 in 1X PBS, pH 7.2, with 5mM NaN_3. Filter through a 0.2 mm filter.