Antibody Titration for Flow Cytometry

Introduction

Antibody titration is performed to ensure that the antibody concentration used is suitable for the experiment. On the one hand, too low an antibody concentration results in insufficient staining of the antigen. On the other hand, too high an antibody concentration will lead to:

- non-specific staining (false-positives, high background, insufficient separation between positive and negative populations → always include a negative sample in your titration experiment)
- unnecessary spill-over (causing problems with compensation)
- unnecessary cost

» **The aim of antibody titration is to find the optimum concentration at which binding saturation occurs.**

Please note that antibody titration:

- must be performed for each device, as the device configuration (laser power and detectors) influence how well different fluorochromes can be detected (i.e. with one device you can achieve a very good separation between positive and negative cells, while with another device you will hardly be able to separate positive and negative cells)
- should be performed under the same conditions as in the experiment (e.g. cell type, blocking, incubation time, temperature, volume etc.).
- should be repeated for each new antibody lot and after each significant change in sample preparation

» **Please note that the cell count is not relevant as long as you are at saturating concentration (in this case 5-fold variations in total cell count have minimal effect on staining). Always stain in the same staining volume, usually 50 - 100 µl, and add the amount of antibody that you optimized by titration for each antibody.**

How to Titrate

- To perform a serial dilution, prepare 8x 1.5 ml tubes (or wells in a microtiter plate), with 100 µl staining buffer in the first tube and 50 µl in all other tubes.
- Add the amount of antibody required for the highest concentration to the first tube. Start with the recommended concentration or slightly higher. Usually 0.1 – 5 µg/ml antibody is used.
- Vortex. Transfer 50 µl from tube 1 to tube 2, vortex, and repeat the procedure until tube 7, discarding 50 µl from this tube.
- Leave tube 8 without antibody. This sample is used as the background control.
- Add 50 µl of a cell suspension to all of the tubes, so that they all contain 100 µl. Use a total number of 2 x 10^5 – 1 x 10^6 cells per sample.
- Stain as usual, e.g. 30 min on ice in the dark.

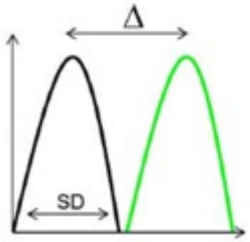
- *Optional*: It is recommended to add a viability dye, as dead cells can bind antibodies non-specifically, making it difficult to analyze the results. If you add a viability dye, make sure to include completely unstained cells as a control in addition to cells stained only with viability dye.
- Wash the cells before proceeding with the analysis.

Flow Cytometry Analysis

1. Run the highest concentration and assure, that the positive fluorescence does not exceed the higher limit of the linearity. If this is the case, reduce the PMT voltage to bring the positive fluorescence slightly below the upper limit of linearity.
2. Run the remaining samples with the same settings. Collect data compromising at least 10,000 live single cells for each concentration.

» **You may need more events if you are looking for a very rare population. It is important to have a sufficient number of positive and negative events. This can be difficult when staining homogenous cell lines.**

3. First gate on the viable cells and exclude doublets.
4. For populations that show a clear positive or negative peak on a histogram or a dot plot, place a marker around the positive signal and another around the negative signal. You must adjust the gates for each dilution.
5. Determine the mean fluorescence intensity (MFI) of the positive and negative populations and the standard deviation (SD) of the negative population. Calculate the Stain Index Δ (SI): *(Pos population Median FL – Neg population Median FL) / 2*Neg population rSD*



$$\frac{MFI_{pos} - MFI_{neg}}{2*rSD_{neg}}$$

6. Determine the optimal antibody concentration as shown in *Figure 1*.

» **The better the distinction between the positive and negative population, the higher the SI.**

When the antibody is increased, the staining index decreases due to the increase in background binding. On the other hand, the staining index decreases as the concentration decreases because the antibody does not reach a saturation concentration.

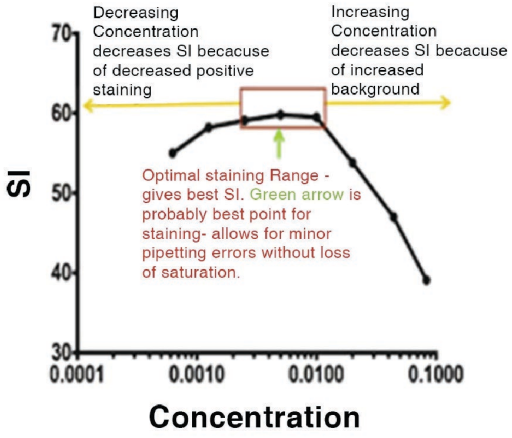


Figure 1 (c) Leinco Technologies, Inc.

Additional Reading

https://www.bio-rad-antibodies.com/flow-antibody-titration.html

https://www.leinco.com/facs-titration/

https://expert.cheekyscientist.com/experimental-optimization-i-determining-the-optimal-antibody-concentration/

IF YOU HAVE ADDITIONAL QUESTIONS, PLEASE CONTACT THE CYTOMETRY CORE FACILITY AT ZMF-CF-Zytometrie@jku.at