Titration of antibody concentration in Flow Cytometry

Written by: Anni Skovbo
Date: May 2020

This guideline work in connection with the FACS Core Facility Guidelines
- Panel Design in Flow Cytometry (7)
- Controls in Flow Cytometry (8)
- Compensation in Flow Cytometry (9)

Why should you titrate?

Titration is performed to assure that you work with the antibody concentration that is appropriate for your experiment.
Too low concentration will of course result in insufficient staining of the antigen.
Too high concentration may cause unspecific staining (false positives, high background), unnecessary spill over (causing trouble doing compensation) and unnecessary cost.

Issues to consider

Titration of antibodies should be performed under the same staining conditions (e.g. time, temperature, lysing, fixing) as you will use in your experiment.

- If you need blocking (6) in your experiment you must also block in your titration experiments.
- You may add a viability stain to your titration samples to be able to exclude dead cells from the analysis.
- Each sample should be stained in the same total volume (sum of cell suspension, viability stain, blocking reagent and antibody dilution).

A titration should be repeated for any given antibody when you receive a vial of the same antibody with a different lot number.
A titration is dependent on instrument configuration. Laser power and detectors influence how well you detect different fluorochromes (meaning on one instrument you might get a very nice separation between positive and negative cells, whereas on another instrument you will hardly be able to separate positive and negative cells).

You need of course positive cells for your titrations, but also negative cells to be able to reveal unspecific staining.

Perform the staining according to your staining protocol considering the abovementioned issues

Perform a serial dilution of your antibody e.g. 2-fold or 3-fold.
If you use 3,16-fold ($\sqrt{10}$) you will get: Undiluted* - 1/3.16 - 1/10 - 1/31.6 - 1/100 - 1/316 - 1/1000 ☺
For the negative control, use staining buffer instead of antibody.
*Undiluted will typically be what is suggested in the antibody's data sheet or one fold higher.

Guidelines for running titration samples on the flow cytometer

(This section is only relevant if you adjust PMT-voltage or gain on the flow cytometer)
During run of your titrations, you must determine and set the appropriate PMT-Voltages for each fluorochrome.
Run your negative control. Set the PMT-voltage to place the negatives a little above the lower limit of linearity
Then run the highest concentration and assure, that the positive fluorescence does not exceed the higher limit of the linearity. If it does you must reduce PMT-voltage to place the positive fluorescence a little below the higher limit of linearity.

How to determine which antibody concentration is better for your experiment

To be able to quantify the quality of the staining, you need to calculate Stain Index (SI):
(Pos population Median FL – Neg population Median FL)/2*Neg population rSD (5)

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

The better discrimination between the positive and negative population, the higher SI.

Calculating SI is a recognized way to determine optimal antibody concentration for each antibody (1).
If you want to reach saturation to quantitate antigens, you choose the antibody concentration resulting in the highest SI.
You may see that even the pos population gets more positive according to higher antibody concentration, the Stain Index gets lower due to a higher unspecific stain of negative cells.

If you just want to know whether the cells are positive you can choose to use less antibody as long as it provides clear separation in between negative and positive cells.
This can save antibodies and besides it may reduce compensation challenges. This is therefore recommended for multi-color panels for the antibodies only used for characterization (2).

Remember to observe the median of the negative population. If it increases, it is a sign of unspecific binding due to antibody overload.

Consequences of the titration results for your PMT-V settings

(This section is only relevant if you adjust PMT-voltage or gain on the flow cytometer)
If you, after performing the titration data analysis, choose to use a lower concentration of antibody, you must reconsider the PMT-V, if the negative population is not above the lower linearity limit.
Run the antibody concentration, you want, and optimize (raise) the PMT-V.
Remember to apply this new PMT-V value to the experiment for your final analysis.

References:

1) https://www.biolegend.com/ja-jp/blog/the-stain-index-what-is-it-and-what-does-it-tell-you
2) This site shows how to find the ”just enough” method: https://www.slideshare.net/PratipChattopadhyay/cyto-2015-forensic-flow-tutorial e.g. slide 29-32
3) BioRad site about titration and more: https://www.bio-rad-antibodies.com/flow-antibody-titration.html
4) Hulspas: Cytom. 2010 Oct;Chapter 6:Unit 6.29. doi: 10.1002/0471142956.cy0629s54.
5) Stain Index: Maecker et al. 2004, Cytometry Part A. 2004 Dec;62(2):169-73.doi: 10.1002/cyto.a.20092
6) Blocking: Andersen et al. 2016, https://www.ncbi.nlm.nih.gov/pubmed/27731950
7) FACS Core Facility Guidelines, Panel Design in Flow Cytometry
8) FACS Core Facility Guidelines, Controls in Flow Cytometry
9) FACS Core Facility Guidelines, Compensation in Flow Cytometry