Antibody titration assay*

To produce interpretable histograms and dot plots, the proper titration of antibodies is recommended. Optimising antibody concentration reduces background noise due to non-specific binding and also significantly reduces costs. In principle, antibodies are diluted serially and used in a single-staining assay. Mean fluorescence intensity is plotted against antibody amount to draw the titration curve.

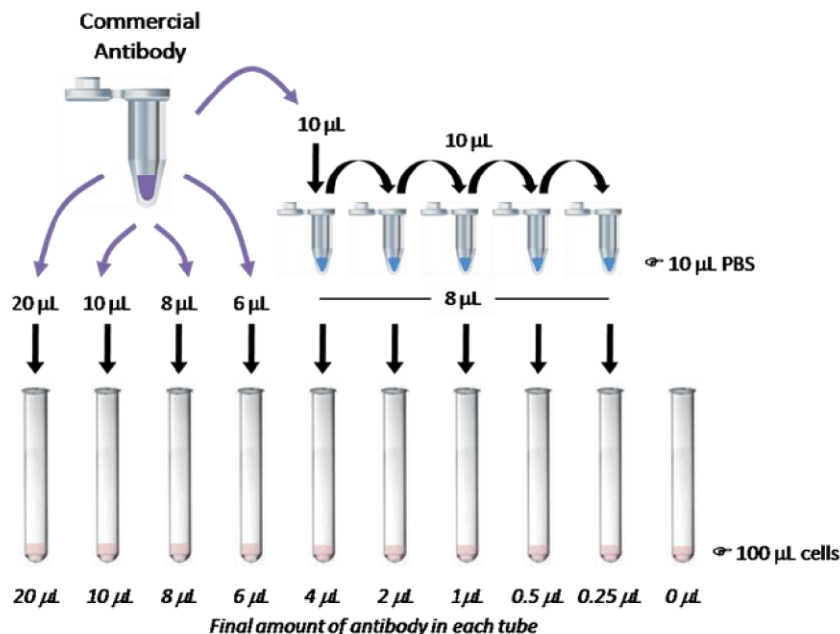
Protocol

This protocol is given for an antibody whose recommended working volume is 20 µL (for 100 µL of cells). This volume was selected as a starting point for subsequent dilutions. Adapt this protocol to any other antibody by adjusting the starting point to the manufacturer's recommendation.

1) Fill 10 flow cytometry measurement tubes with 100 µL cell suspension.
2) Fill five microtubes with 10 µL phosphate-buffered saline.
3) Dilute the commercial antibody serially in microtubes by carrying 10 µL each time to the next tube (mix well and change pipette tip). See table below.
4) Add decreasing antibody volumes of commercial antibody stock (from 20 µL down to 6 µL) to the cell suspension.
5) Add 8 µL diluted antibody to the cell suspension.
6) Incubate, wash and acquire as usual.

Volumes, µL	Decreasing volume				Serial dilutions				
Antibody	-	-	-	-	10	10[a]	10[a]	10[a]	10[a]
PBS	-	-	-	-	10	10	10	10	10
Stain added to 100 µL cell suspension	20	10	8	6	8	8	8	8	8
Final amount	20	10	8	6	4	2	1	0.5	0.25

[a]Pipette from previous tube; dash indicates non-applicable
PBS, phosphate-buffered saline



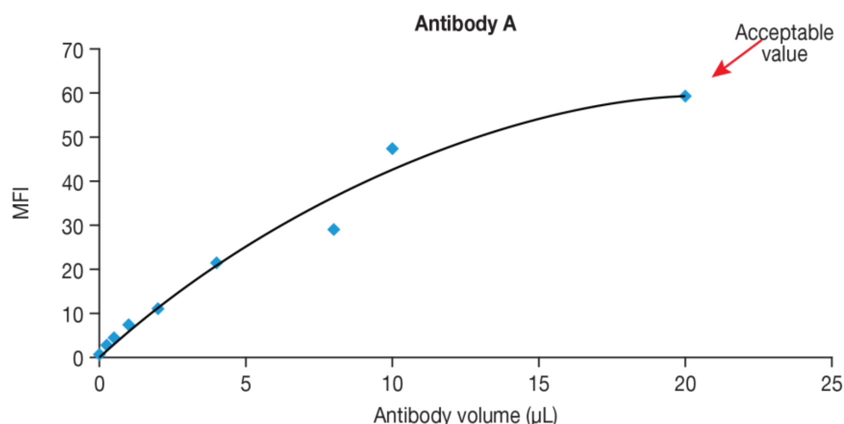
PBS, phosphate-buffered saline

Determining quantity of antibody to use in assay

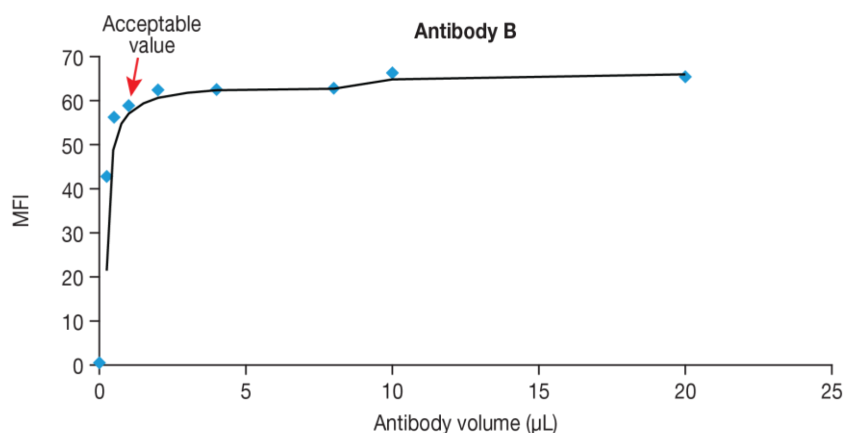
To determine optimal antibody quantities for flow cytometry assays, use the lowest concentration of antibody that results in at least 80% of the maximum signal intensity. By reducing the antibody concentration, background noise due to non-specific binding is reduced (as well as saving on cost).

- **CD235a (glycophorin A [GPA]):** The plateau is not reached even when using 20 µL of antibody, which is the amount recommended by the manufacturer. Given the shape of the dilution curve, using less antibody is not advisable.
- **CD59:** As low as 0.5 µL of antibody yields 85% of the maximum staining intensity. This would be perfectly acceptable. However, to avoid dilution effects for samples that are highly cellular, a safety margin must be applied. Between 1–2 µL of antibody should be used (eg 1.5 µL).

Antibody A	
Antibody volume (µL)	MFI
0	0.62
0.25	2.73
0.5	4.41
1	7.35
2	11.00
4	21.39
8	28.96
10	47.31
20	59.23



Antibody B	
Antibody volume (µL)	MFI
0	0.45
0.25	42.73
0.5	56.16
1	58.86
2	62.35
4	62.45
8	62.75
10	66.22
20	65.34



MFI, mean fluorescence intensity

*These protocols were developed in close collaboration with Mrs Andrea Illingworth of Dahl-Chase Diagnostic Services in Bangor, ME, USA, Drs Thomas Matthes and Mathieu Hauwel of the Swiss Flow Cytometry School at the University Hospital of Geneva, Switzerland, and Dr Iuri Marinov of Hematology and Blood Transfusion in Prague, Czech Republic. Images were provided with permission from the netflow Steering Committee and Swiss Flow Cytometry School.