ICCS
International Clinical Cytometry Society

Sponsored and reviewed by ICCS Quality and Standards Committee

Title: Quality of Reagents – Monoclonal Antibodies

Written by: Ruud Hulspas, Mike Keeney, Ben Hedley and Andrea Illingworth

Date: March 6, 2018

OUTLINE:

In clinical flow cytometry, monoclonal antibodies should be validated in the context of the assay as part of the assay validation procedure. It is highly recommended to use well described monoclonal antibodies derived from clones described by the Human Leukocyte Differentiation Antigen (HLDA) Workshops. Reference material is used to validate the reactivity, specificity, selectivity and sensitivity of an antibody. The type of reference material is determined by the assay and may be comprised of (in order of preference if the material is available) 'normal' cells from 'healthy' donors, a known positive cell line, or other quality control material including commercially available QC material. A titration assay is used to verify antibody reactivity and specificity, and also to determine the antibody amount and concentration resulting in the lowest level of non-specific binding and the highest amount of specific binding.

Regulatory concerns: Most clinical US laboratories use either FDA approved IVD (in vitro diagnostics) or ASR (analyte specific reagent) antibodies. The use of Research Use Only (RUO) reagents are not for use diagnostic or therapeutic practices in the United States and should be limited to research applications in the United States and Europe. These reagents are not to be used as the sole parameter to establish a diagnosis or decide on a therapeutic treatment, and require internal validation at the user's facilities with clinical samples.

PROCEDURE/PROCESS

Identify the target antigen and ensure the following basic information about the antibody product is documented:

- Name (approved, alternative names)
- Clone
- Isotype
- Conjugate
- Manufacturer and Catalog#
- Lot#
- Reactivity / Cross reactivity
- Formulated Concentration
- Qualified Applications (live, fixed, fixed-permeabilized)
- Recommended Storage
- Brief description of target antigen
- Regulatory status IVD (Europe CE), ASR, RUO (GMP) or RUO)
- References

Assessing the optimal amount of antibody through titration:

Confirm reactivity and specificity by means of an antibody titration assay, using reference cells known to contain cells that do, as well as cells that do not express the target antigen.
All titrations should be performed under the same test conditions that will be used in the final assay. Key points are cell concentration, staining buffer and reaction volume.

- With a Manufacturers Recommended Volume: Not all antibodies from any single supplier will come with a recommended user volume, however if the antibody under investigation does have a suggested volume then this is an ideal place to start. Using the recommended volume followed by using serially lower amounts of antibody. If the antibody is still dim at the recommended volume then a tube with double the recommended volume may be set up. This should cover a range that will yield an appropriate volume of this antibody to use in diagnostic testing.
  Example: If the manufacturer's recommendation is 5 µL of antibody for 1 million cells in a total assay volume of 100 µL, then this is the starting point for the dilution series. The subsequent serial dilutions will contain 2.5 µL, and 1.25 µL of the original antibody solution. The titration series is completed by preparing a tube containing 10 µL of the original antibody solution (2-fold higher than the recommended amount) and a tube with no antibody ('unstained' sample).
- Without a Manufacturers Recommended Volume: If no recommendations are available, it is best to expand the titration set from 5 to 7 and use 0.2 µg as a generic amount of antibody as starting point. The volume of antibody solution that contains 0.2 µg of antibody can be calculated from the formulated antibody concentration of the product. Once established, this preparation can be used to prepare 3 subsequent 2-fold serially diluted samples (0.1 µg, 0.05 µg and 0.025 µg,). Complete the titration series with samples containing a 2-, and a 4-fold higher concentration, and a tube that does not contain antibody.
  Example: If the formulated antibody concentration of the product is 100 µg/mL, the antibody volume for the starting point of the serial dilution (0.2 µg) is 2 µL. The volumes to use to prepare the subsequent dilutions contain antibody amount equivalent to 1 µL, 0.5 µL and 0.25 µL of the original product, respectively. The titration series is completed by preparing a tube containing 4 µL of the original antibody solution (2-fold higher than the recommended amount), a tube containing 8 µL (4-fold higher than the generic amount), and a tube with no antibody ('unstained' sample).

Developing acceptability criteria for antibody titrations

Visual assessment by looking at plots provides invaluable information on how well the target population separates from other populations. There are often details in this visual assessment which cannot be entirely provided by numerical values such as spread or bimodal distribution of the target population, non-specific fluorescence on other than the target population, and other details of antigen expression. However, both the objective measures (e.g. Median Fluorescence Intensity, MFI) and the visual assessment are necessary to assess antibody performance and define standards of acceptability. As with many applications in flow cytometer both of these measures of antibody performance must relate back to the assay in which the antibody is to be used. Each antibody is different and depending on the assay the laboratory needs to develop criteria for acceptability. For instance, the brightness (MFI) of CD3 may be sufficient for the quantitative assessment of CD3+/CD4+ populations while the more qualitative assessment of a possible decrease in CD3 staining in certain T-cell malignancies might require different criteria of acceptability for this antibody.

There are different ways to assess the quality of an antibody:

1. Relative intensity of MFI: For this assessment the MFI of the antigen-positive population is divided by the MFI of the antigen-negative population. This works well for the basic assessment for most assays but does not take into account the "spread" of a population. This can be seen is the standard deviation (SD) of the negative population is measure during acquisition.
2. Staining Index (SI): To assess the lowest level of non-specific binding and the highest level of specific binding, the fluorescence intensity of a 'negative' cell population is compared to that of cells in the unstained sample, while the optimum separation between a 'negative' and 'positive' population of cells is determined by calculating the Staining Index[1]:

$$\textbf{Staining Index} = \frac{(Median_{pos} - Median_{neg})}{2\ x\ (robust\ SD_{neg})}$$

Staining Index (SI)[2] values obtained from each sample in the titration series can be plotted in a graph against values that represent the respective antibody concentration (and/or amount). In general, this results in a series of data points through which a curved line can be drawn with the maximum SI value as the 'top', displaying the best antibody concentration and amount to label representative cell sample. Reason for using the staining index is to normalize the signal to the negative population spread which tends to get broader with higher antibody concentrations. Robust SD_{neg} is calculated as the average of the Standard Deviations of all the negative populations (SD_{neg}) run in the titration series.

Although the SI is a good tool to determine maximum separation between the relative intensities of two cell populations labeled with the same fluorochrome, it provides little information about the actual level of non-specific antibody binding. The assessment of non-specific antibody binding is beyond the scope of this article, however briefly it can be calculated from comparing the MFIs of known negative populations in stained samples against an unstained sample. Example of this is examining the B cells (CD3-negative) in samples stained a titration series for CD3.

Due to instrument variability the value of the SI is arbitrary and cannot be used to compare separations measured by different detectors, nor instruments.

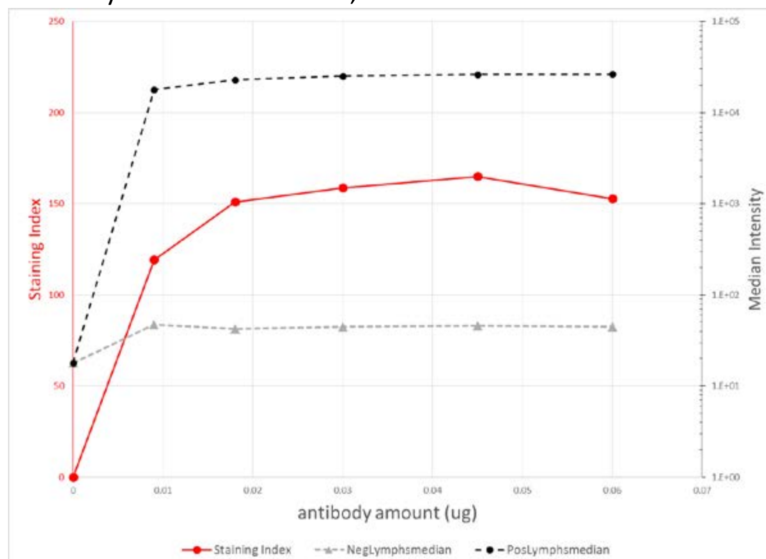

Figure 1 - Example of staining index. The median value for the negative population, the positive population and the SI are shown.

After antibody reactivity and best labeling conditions have been established, antibody specificity should be confirmed within the parameters of the intended assay by assessing antibody binding to cell populations used in the assay that are known to not express the target antigen.

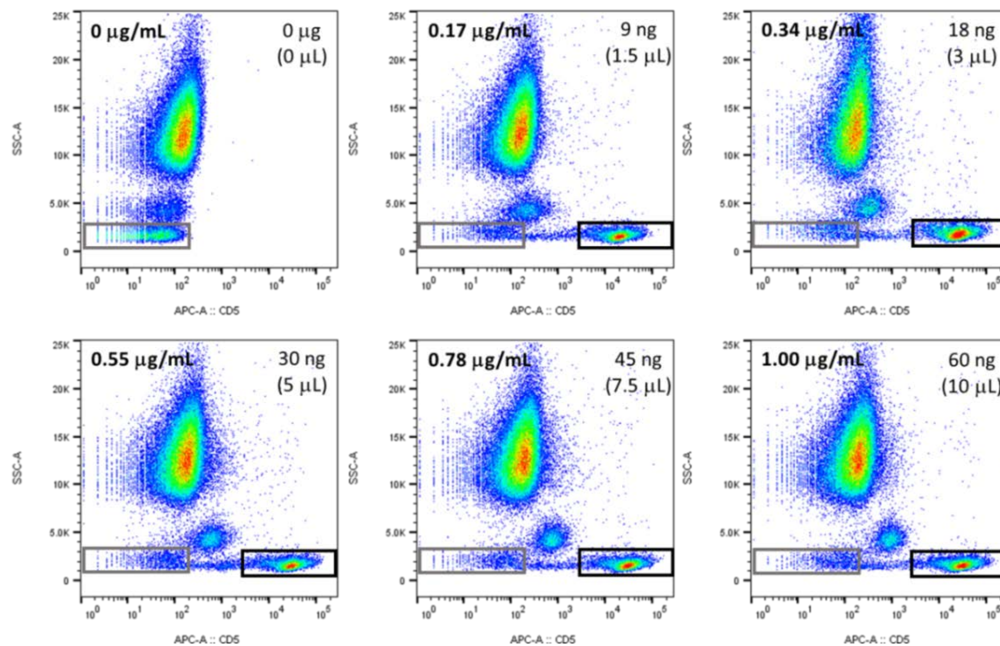

Figure 2 - Titration of an APC-conjugated mouse monoclonal IgG2a antibody against human CD5 (clone L17F12, formulated at 6 µg/mL, ASR, BD Biosciences) using approximately 0.5 million cells per tube. In this example, the use of blocking buffer (e.g. Bovine Serum Albumin or commercial product) was omitted to show increase in unwanted binding (to monocytes). Each plot indicates the final antibody concentration (in bold displayed in left upper corner) and how much antibody was used in nanograms as well as in microliters taken from the vial (displayed in right upper corner). The grey and black regions were used to determine the median fluorescence intensities of the 'negative' and 'positive' cell populations, respectively. Manufacturer's recommended antibody volume was 5 µL.

Below are some practical examples of antibody titrations. Each antibody is different and based on the assay, the laboratory needs to determine what the criteria for acceptability are. For some antibodies, a basic comparison of relative MFI (S/N ratio) may be sufficient, while other antibodies require a more in depth evaluation and a staining Index assessment may be more appropriate.

A - Basic titration: Using relative intensities of MFIs (S/N) and visual inspection of dotplots:

Most titrations are performed by using a plot with the fluorescence intensity on the x axis and side scatter (SS) on the y axis. The voltage should be adjusted so both the unstained (or antigen-negative population) and the antigen-positive population are clearly visible as clustered populations. If the PMT voltage is too low the antigen-negative population cannot be seen on the scale and is too far to the left. Conversely if the PMT voltage is too high, the antigen-positive population also cannot be seen on the x axis and is too far to the right (too bright).

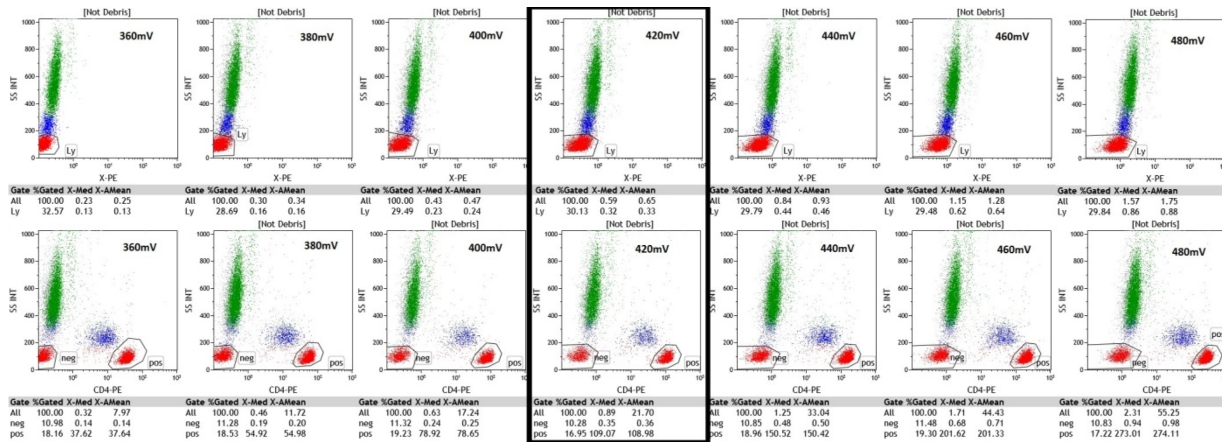

Figure 3 -Voltage adjustment: Top row shows unstained cells at different voltages, the bottom row shows cells stained with CD4-PE at the same voltages. The plots to the left of the black box show suboptimal voltages (too low), the plots in the black box show an optimized voltage setting to show all populations "on scale" on a 4 decade log scale while the plots to the right show suboptimal voltages (too high).

<u>Basic procedure:</u>

1. Label tubes as follows:
    a. Appropriate number of tubes ("Antibody + lot#" + volume [or concentration]) stating the recommended amount and amounts based on the manufacturers recommended volume or calculated volume from the concentration (e.g. 10µL). Suggested amounts include the recommended volume (10µl) followed by lower amounts 7.5µL, 5 µL, 2.5 µL and 1.25 µL)
    b. "Unstained" tube.
2. Add cells or sample, and antibody to be titrated
3. Incubate for 20 minutes in the dark at RT
4. Lyse the red blood cells (if required for the assay) and wash the sample (if required by the assay)
5. Acquire on flow cytometer with assay-specific settings for PMT voltages (clear compensation and do not use baseline offset for titrations)
6. Analyze median fluorescence vs side scatter of the positives and negatives (use gating that tightly gates the populations of interest)
7. Calculate the S/N for each concentration
8. Graph median MFI and /or signal/noise ratio versus the concentration used

An example of CD24-PE titration is shown below and in order to determine the <u>acceptability criteria</u>, one has to consider the expected antibody performance for each specific assay. For instance, in the PNH assay, CD24 staining needs to be fairly bright and show good separation between CD24-negative PNH neutrophils and CD24+ normal neutrophils to allow for accurate detection of PNH clones. Ideally, a PNH sample should be used for this titration; however, PNH samples are rare and normal blood is used as an alternative sample for this titration.

Important criteria for the CD24 antibody performance in a PNH assay include:

1. Tight clustering of the CD24+ neutrophils based on visual inspection of the SS vs FL-2 (PE)
2. Good separation (S/N) between CD24-negative lymphocytes and CD24+ B-lymphocytes
3. Good separation (at least one log decade) between CD24-neg lymphocytes and CD24+ granulocytes

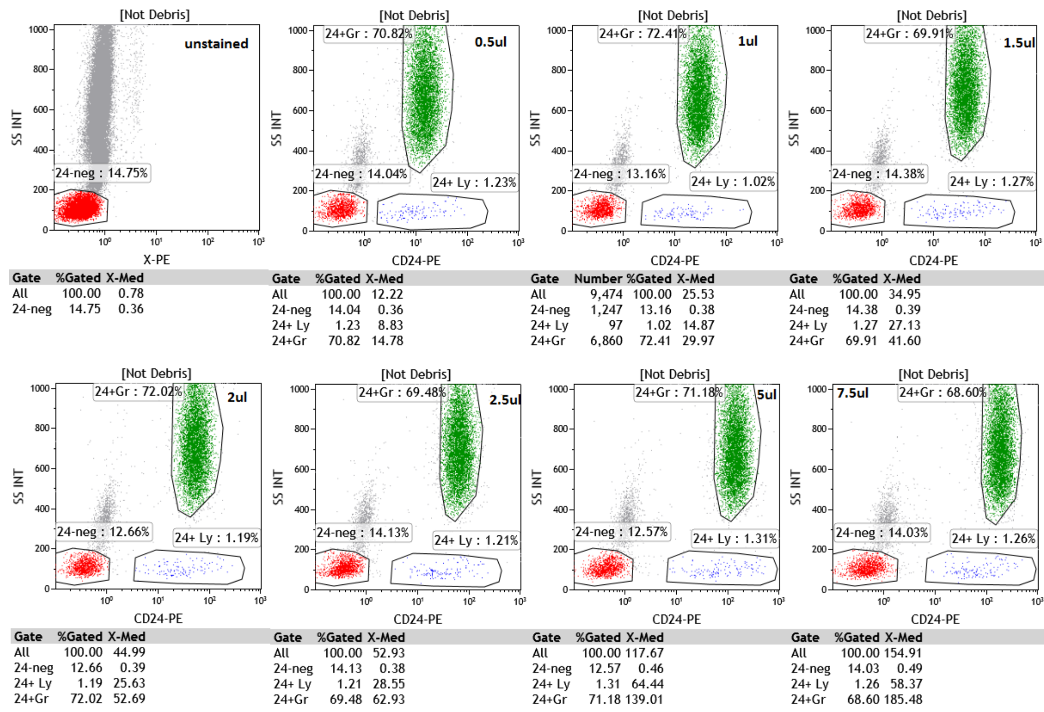

Figure 4 – Example of titration of CD24-PE (clone SN3, ThermoFisher). Recommended amount of antibody is 5µL/0.5 million cells. The table below shows the highest signal to noise ratio at 5ul/test (yellow). However, based on a combination of visual inspection and the numerical values, the performance at 2.5ul/test is acceptable (orange).

	unstained	0.5ul	1ul	1.5ul	2ul	2.5ul	5ul	7.5ul
MFI CD24-neg	0.36	0.38	0.39	0.39	0.39	0.38	0.46	0.49
MFI CD24+ Ly		8.8	14.9	27.1	25.6	28.6	64.4	58.4
MFI CD24+ Gr		14.8	30.0	41.6	52.7	62.9	139.0	185.5
S/N ratio (lymphs)		23	38	70	66	75	140	119
24+Ly/24+Gr ratio		39	77	107	135	166	302	379

1. In this scenario, CD24 is used for PNH testing and it is important to get the best clustering of the CD24+ neutrophils as well as the best signal/noise ratio in order to separate CD24+ normal neutrophils from abnormal CD24-negative PNH neutrophils. In this example CD24 is conjugated to PE.
2. A visual inspection of the SS vs FL-2 (PE) is used in this case to assess the appropriate location of each relevant population in addition to comparing the MFIs of the CD24+ neutrophils as well as the CD24 negative lymphocytes vs CD24+ B cells.
3. When comparing the titrations below visually there are is no clear volume that appears to perform better than another. All examples shown in Figure 4 show good separation of the CD24 positive neutrophils from the negative staining lymphocytes.
4. When calculating the signal to noise the suggested volume for the CD24 antibody would be approximately 2.5 µL/test.
5. It should be noted that the MFI of the negative population does not significantly change which increasing volume of reagent.

B - Using MFI and Staining Index (SI)

In the example below (Figure 4), the use of blocking buffer was omitted. Each plot indicates the final antibody in microliters taken from the vial (displayed in right upper corner)..

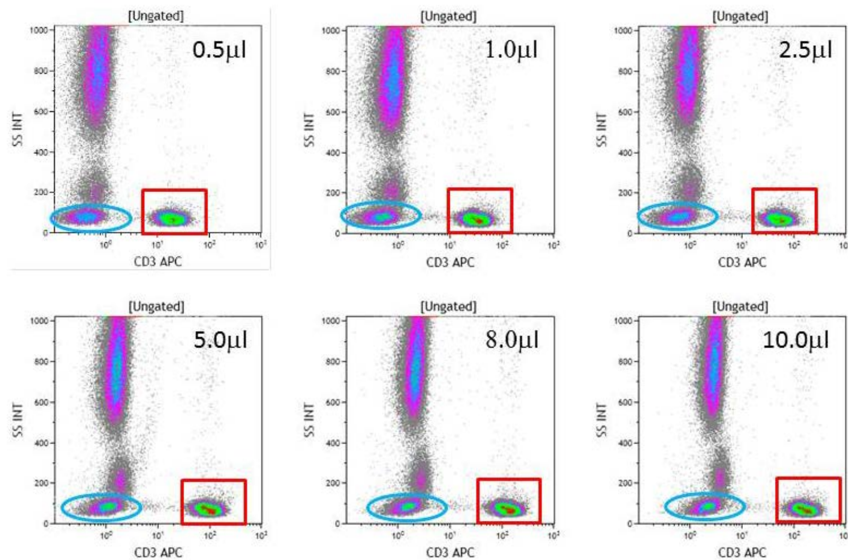

Figure 5 -Example of a titration of mouse anti human IgG1 CD3 conjugated to APC (clone UTCH1, Beckman Coulter Inc.). The red and blue regions were used to determine the median fluorescence intensities of the 'negative' and 'positive' cell populations, respectively. Manufacturer's recommended antibody volume was 10µL

Volume	MFI+	MFI-	SD_{neg}	S/N	SI	% Gated
10 uL	160.088	2.178	1.215	73.5	100.3	70
8 uL	127.259	1.537	1.055	82.8	113.0	70
5 uL	92.462	1.075	0.859	86.0	117.4	70
2.5 uL	52.466	0.61	0.502	86.0	117.4	70
1 uL	32.016	0.492	0.492	65.1	88.8	70
0.5 uL	19.258	0.422	0.274	45.6	62.3	70

Table 1 - Data table showing the Median fluorescence for each of the volumes used in the titration of CD3 APC.

Average of $SD_{negative}$ = 0.733

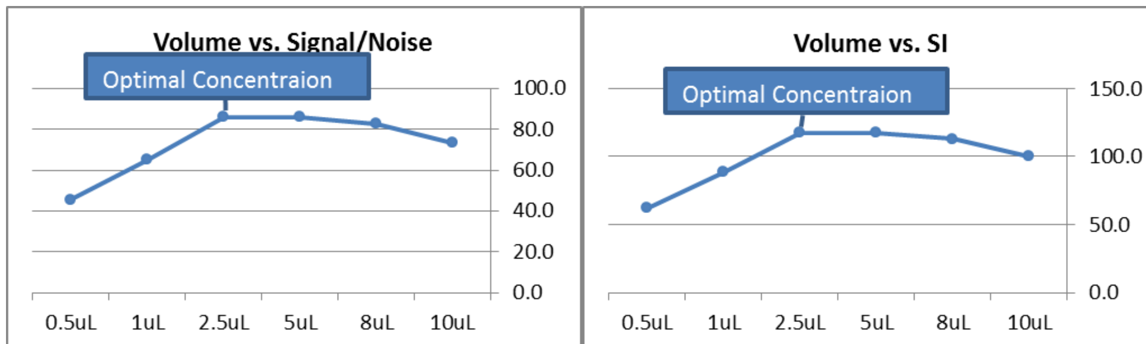

Figure 6 - Example of the CD3 APC titration showing in A) the volume versus the Signal to Noise calculation and B) the Staining Index (SI).

In the example above for CD3 APC the SD for the negative population (B cells) in the titration can be seen to increase 6-fold. For this reason the SI should probably be used to calculation the optimal volume of antibody to staining for CD3 positive cells. In this example both the signal to noise and the staining index plotted both against the volume and it can be seen that the optimal volume is the same however this may not always be the case.

SUMMARY

Assessing the antibody performance is a required component in any flow cytometry laboratory. It is important to understand that the vendor is only responsible for the quality of the reagents but the laboratory is responsible for developing assay-specific criteria for acceptability for each reagent/antibody for its intended use. The readers should be aware of antigen/antibodies that are harder to titrate because of their distribution; CD38 is an example of this. It is a key reagent in many clinical flow assays (as well in research), however titrating CD38 is very difficult because of lack in clearly definable internal negative control populations.

Titrations and lot-to lot comparisons (see reference 5 for lot to lot comparisons) are an important factor in the internal assessment of the laboratory's currently used antibodies and communication with other laboratories is often helpful in developing a better sense of what is an "acceptable" versus "suboptimal" antibody. The purpose and scope of this module was to provide basic guidance for titration. There are many aspects to assessing the quality of an antibody and a titration is just a first step in determine the overall antibody performance. It should be noted that not all antibodies can be assessed by simple S:N/SI graphs because internal negative/unreactive cell populations are not always present. Future modules will address each of the key antibodies for each assay and discuss their assay-specific performance criteria in the context of the intended use.

REFERENCES:

1. Maecker T, Frey T, Nomura LE, and Joe Trotter J. Selecting Fluorochrome Conjugates for Maximum Sensitivity. Cytometry 2004; 62A: 169-173.
2. Bigo M. Separation Index: An Easy-to-Use Metric for Evaluation of Different Configurations on the Same Flow Cytometer. *Current Protocols in Cytometry*. 2007; 40: 1.21.1–1.21.6.
3. Davis BH, Wood B, Oldaker T, Barnett D. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS, Cytometry B Clin Cytom 2013 84B: 291-308
4. ICCS Question from ICCS Educational committee: "What is MFI and how is it calculated?" by A Illingworth and PK Wallace (2017)
5. Sutherland DR, Illingworth A, Marinov I, Ortiz F, Andreasen J, Payne D, Wallace PK, Keeney M. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders part 2 - reagent selection and assay optimization for high-sensitivity testing. Cytometry B Clin Cytom. 2018 Jan;94(1):23-48.

Reviewed and Approved by:

1. Brahmananda R. Chitteti
2. Rob Sutherland

The documents posted on ICCS website may contain product or vendor names which are provided for platform specific guidance. Any reference within the ICCS Quality and Standards modules to any vendor, product or educational material by trade name, trademark or manufacturer does not constitute or imply the endorsement or recommendation by ICCS.