REVIEW ARTICLE

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Relevance of Antibody Validation for Flow Cytometry

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Abstract

Antibody reagents are the key components of multiparametric flow cytometry analysis. Their quality performance is an absolute requirement for reproducible flow cytometry experiments. While there is an enormous body of antibody reagents available, there is still a lack of consensus about which criteria should be evaluated to select antibody reagents with the proper performance, how to validate antibody reagents for flow cytometry, and how to interpret the validation results. The achievements of cytometry moved the field to a higher number of measured parameters, large data sets, and computational data analysis approaches. These advancements pose an increased demand for antibody reagent performance quality. This review summarizes the codevelopment of cytometry, antibody development, and validation strategies. It discusses the diverse issues of the specificity, cross-reactivity, epitope, titration, and reproducibility features of antibody reagents, and this review discusses the validation principles and methods that are currently available and those that are emerging. We argue that significant efforts should be invested by antibody users, developers, manufacturers, and publishers to increase the quality and reproducibility of published studies. More validation data should be presented by all stakeholders; however, the data should be presented in sufficient experimental detail to foster reproducibility, and community effort shall lead to the public availability of large data sets that can serve as a benchmark for antibody performance. © 2019 International Society for Advancement of Cytometry

Key terms

flow cytometry; monoclonal antibody; validation

Flow cytometry has developed into an indispensable technique in the research and clinical investigation of immune and hematologic systems, with increasing applications in other cell biology disciplines. There are three main pillars in the practical application of this technology, namely the instrumentation, the analytical methods for large data sets, and the reagents used to design biological experiments. Despite dramatic developments in instrumentation (polychromatic (1), mass (2,3), and spectral (4,5) cytometry) and the significant developments in data analysis techniques for high content analyses (6–8), the last pillar of this trio remains consistent across time in its importance to the application: the fluorescent antibody conjugate.

Our and others' experiences indicate that nearly half of antibodies, sold by companies or described by academic groups, do not function for the recommended application. They present staining patterns that conflict with those reported in the literature, show unexpected cross-reactivity, or have even failed the most basic specificity tests (9–11). There is growing alarm about results that cannot be reproduced by other research groups, including data published in high-impact journals. Antibodies are believed to be, in part, responsible for inconsistent experimental results and the publication of inaccurate data in the scientific literature (12,13).

During recent years, both industry and academic groups have increased their efforts to increase the quality of the validation process of antibodies and to provide the validation data of their antibodies to the user community. However, there is no consensus on the level of validation by manufacturers and how this information should be disseminated. Moreover, novel methods of antibody validation have

emerged that offer a deep and more definite assessment of target identification. Antibody producers face the problem that some of these methods are quite expensive, and the increased demand for high quality validation has to be balanced with the cost of the antibody.

Importantly, choosing the best antibody is not easy to figure out for the user, especially keeping in mind that there are more than 300 estimated antibody suppliers (9).

Thus, the aim of this article is to describe the basic principles of antibody validation for flow cytometry and to discuss which methods for antibody validation are preferable, acceptable, and feasible. It is of the utmost importance to the field to reach a higher quality in validation, reproducibility, and reporting of antibody-based cytometry findings.

What Have we Achieved with Antibody Reagents in Cytometry?

Cytometry applications have grown from a single color, single set of sample evaluations into 8–10 color clinical (14), 19-color spectral (15), 28-color polychromatic (16,17), and 42 mass (18) cytometry applications over the four decades of its evolution. Reagents with reliably consistent molecular brightness, coupled with the use of consistently sensitive PMTs, offer the benefit of reproducible signal intensity quantitation. This allows us the consistency needed for the meaningful measurements over long periods of time (month to years) (19,20) that are primarily used for diagnostics and the monitoring of treatment efficacy (21); but they are also increasingly used in preclinical human studies (22). The large data sets that result from these types of immuno-monitoring applications require a method of computational analysis for the resultant large cytometry data sets (7,23–26). A requirement for the successful use of computational methods is that an assumption can be made that any changes detected in the data are related to the biological question (and are not due to technical variation) (8,27). As with any complex interconnected system, a large-scale cytometry experiment is only as good as its weakest component. If this general statement is translated to the validation and performance requirements for antibody conjugates, it is essential that each conjugate in the multicolor panel has an optimal and reproducible performance. The performance criteria of antibody conjugates are application dependent and should be validated as such. While a relatively low level of signal intensity reproducibility is needed for discretely expressed antigens, such as CD4 on CD4+ and CD8 on CD8+ T cells, a much higher intensity reproducibility is needed for variable quantitative measurements, such as an increase in phospo-STAT1 levels after ruxolitinib withdrawal (28). Likewise, computational methods that perform analyses of large cohorts require that identical cells in different timepoints or in different individuals have a precisely the same immunophenotype signal intensity in all measured parameters; hence, antibody conjugates used in different timepoints in different laboratories should have known (and for this purpose identical) performance parameters. This can be achieved with the currently produced reagents. For example, the overall pattern, as well as individual parameter variation, is systematically followed in EuroFlow Quality Assessment, where signal readout variation is as low as 30% (CV of median fluorescence intensity) for 7 of 11 surface proteins with their stable expression evaluated over 4 years in 11 laboratories (20); although reagents of different clones and different manufacturers (carefully selected and tested alternatives for equal signal intensity on target cells) were used. Stringent performance criteria are needed to respect the features of the target protein (stability of expression), particularities of the epitope, nature of the monoclonal antibody (specificity and affinity), and sample preparation protocol (titration and fixation).

Another important movement in the field is the growing number of cytoplasmic and nuclear targets. Those are being detected in complex multiparametric assays in parallel to surface immunophenotyping. This opens a way to understand how different cellular subsets within a sample react to various stimuli ex vivo (the secretion of cytokines by intracellular cytokine staining), how the surface immunoregulatory proteins vary on T-cell subsets (16), which transcription factors play a role in various cell subsets in health and disease (nuclear staining) (29), how complex cellular signals are propagated within particular cell types (phospho-kinases measurements by phospho-flow) (30) or whether a particular protein is abnormally expressed in a given cell type as a result of germinal or somatic mutation (intracellular detection) (31,32). Those assays require antibody monospecificity (only the target is recognized and no other off-target binding occurs) as well as sample preparation techniques (fixation and permeabilization conditions) that would allow for the simultaneous detection of multiple targets; thus, ideally, performance criteria with several sample preparation techniques shall be known about antibody conjugates. The establishment of those assays is tedious because a compromise sample preparation method has to be found to allow for the measurement of all intended targets on a single-cell level in one sample. Unlike in other laboratory techniques, cytometry suffers from a lack of a standard analyte material since engineered particles containing known amounts of analyte are largely unavailable. However, the well-documented expression patterns of target proteins in generally available primary cells or cell lines might play a role as a biological standard useful for antibody conjugate benchmarking.

Thus, at present, multiparametric cytometry assays require antibody conjugates with known performance criteria under several conditions; for several cell types, validation data shall be presented for monoclonal antibody reactivity and for antibody conjugate performance. Consensus on benchmarking methods, aggregation of comparable data sets across manufacturers and users and public availability of the performance data on clones as well as on antibody conjugates will lead to a qualitatively higher level of information generated by flow cytometry and to a further spread of its applications to basic, translational, and clinical research.

Antibody Generation and Cluster of Differentiation Nomenclature

Antibody reagents for flow cytometry were first developed for leukocyte cell surface proteins, usually after immunization

with whole cells and the blind search for targets being recognized. In the late 1970s, immunologists began to generate very large numbers of monoclonal antibodies (mAbs) with the advent of hybridoma technology. A plethora of human cell surface molecules were identified and described within a few years. The problem emerged was that different mAbs produced by several laboratories, under different names, were actually directed against the same molecule. This was not always obvious because the description of the expression pattern reflected differences in local staining techniques and protocols. This produced a nomenclature chaos with different laboratories referring to identical molecules with different names in their publications (33). To avoid confusion, in 1982, the first Human Leukocyte Differentiation Antigens (HLDAs) Workshop was organized to implement a standard nomenclature (34). Since then, the succeeding HLDA workshops have played a crucial role in establishing both target identity and the community supervision of that process, enabling the widespread use of antibodies for cytometry (35).

The basic strategy has been to blindly assess mAb reactivity with a large panel of primary normal and malignant lymphoid cells and cell lines using multiple-color flow cytometry, followed by statistical clustering analysis of the resulting expression data. The mAbs that cluster together are further examined for the biochemical nature and molecular mass of their target molecule by immunoprecipitation. Although cellular expression analysis remains essential, molecular biology techniques, such as the study of transfected cells or the expression silencing, have become essential for the establishment of the target identity. Currently, it is mandatory to exclude the cross-reactivity of the Abs with proteins encoded by a common gene family. This is essential if the degree of homology between molecules of the same family is high. The numbers of CDs have risen dramatically during the 10 HLDA Workshops. At present, CD markers range from CD1 to CD371, with some CDs covering a group of closely related proteins or carbohydrates (e.g., CD1a, CD1b, CD1c, and CD1d) (35). The CD nomenclature is also used to name the molecule itself. For example, CD20 designates both the group of mAbs recognizing the CD20 cell surface molecule and the CD20 molecule itself. HLDA workshops and CD nomenclature have played a crucial role in establishing a global antibody classification scheme, providing consistency in papers that refer to identical molecules. Currently, mAbs are raised against known molecules, especially using recombinant proteins or cells transfected with immunogens. However, HLDA Workshops are not only very efficient in naming antibodies and molecules but also a very effective and comprehensive way to independently validate mAbs, and thus, ensure that developed antibodies can be trusted.

What Issues Do we Face with Antibody Reagents?

Specificity—Is the Intended Target Recognized?

The most important characteristic of a mAb to be used as an analytical tool is its ability to specifically and selectively recognize a unique target molecule. MAbs specifically recognize unique regions of the target molecules called epitopes. Frequently, it is a key to the success of knowing the exact epitope recognized by the antibody; for example, it is helpful to know whether the same epitope is present on all the isoforms of a protein, for example, CD45, or whether it is specific for different isoforms, such as CD45RA or CD45RO (36).

Cross-reactivity—Are Unintended Targets Recognized?

An antibody can present specific reactivity with its epitope, however, similar domains of other molecules might share a sequence identity and thus the antibody would cross-react.

This is especially relevant when studying the expression of proteins that belong to a gene family with different members that present high homology because these proteins will share identical epitopes. Paradigmatic examples of this type of cross-reactivity are antibodies directed against the CD66 (CEACAM) and CD85 (leukocyte immunoglobulin-like receptor (LILR)) families or G-protein coupled receptors (GPCRs), where a high number of antibodies have been shown to recognize different members of the family, generating a large amount of false results (37–39) (Fig. 1).

In some cases, cross-reactivity has been observed in epitopes that are not predictable based on sequence analysis (40). For example, an antibody against a cell surface molecule (HLA-DR) that cross-reacts with an unrelated nuclear molecule (DNA), thus giving false-positive results if dead cells are not properly removed. While most of these antibodies belong to the IgM isotype, in some instances, these antibodies can also be IgG.

While a low degree of cross-reactivity might be acceptable in assays that resolve target protein size (e.g., Western blot), this assumption cannot be applied to flow cytometry (despite claims that it can be controlled by diluting the antibody) since the expression of the off-target epitopes may vary considerably among the analyzed samples. In any case, as we will describe later, an essential goal of a validation protocol is to determine the specificity and selectivity of an antibody and to exclude antibodies that exhibit cross-reactivity.

Epitope nature—Do the Characteristics of the Recognized Epitopes Matter?

Users should be aware that most antibodies that preform perfectly for techniques, such as Western blot (WB) or immunohistochemistry analyses do not work for flow cytometry. This is mainly because these antibodies recognize linear epitopes that are only accessible when denatured. Flow cytometry analyses whole cells and their proteins in their native form. Thus, most of the antibodies that are used in flow cytometry recognize conformational epitopes. This explains why it is important to have information about the epitope recognized by the antibody. It also stresses the point that antibodies for flow cytometry have to be specifically validated for this application and that a perfectly validated antibody for WB (denatured conditions) will not be, in most cases, useful for flow cytometry and immunoprecipitation (native conditions) (for details, see publications by Lund-Johansen et al.(41–43)). In

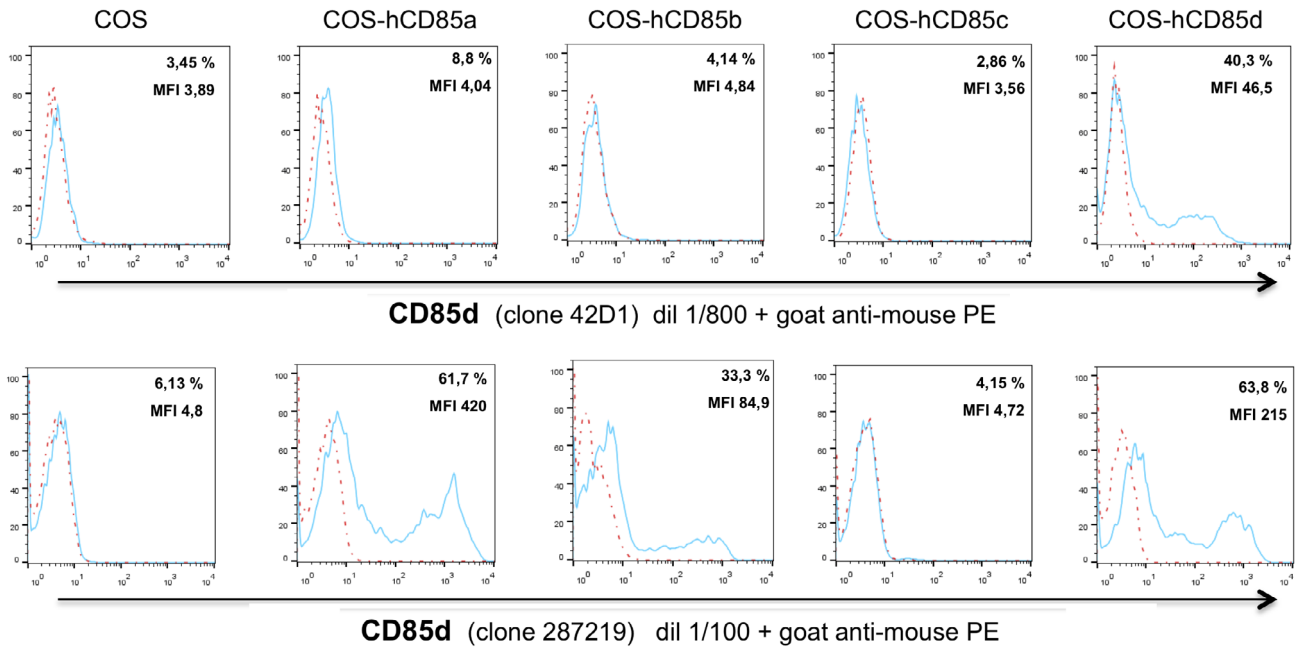


Figure 1. Reactivity of two CD85d clones with COS cells transfected with the cDNA of CD85a, CD85b, CD85c, and CD85d. While the clone 42D1 only stains CD85d and exhibits no off target binding, the clone 287,219 reacts with its target (CD85d) but also cross-reacts with additional CD85 family members (CD85a, CD85b). [Color figure can be viewed at wileyonlinelibrary.com]

addition, negative charge of particular glycosylated molecules (e.g., CD34 antigen) hampers binding of conjugates with negatively charged fluorophores (e.g., FITC) (44). Antibodies to other highly O-glycosylated structures such as CD235a (glycophorin A) can behave in unpredictable ways as well, with PE conjugated forms sometimes causing significantly greater aggregation of RBCs than their negatively-charged FITC counterparts (45). Currently, many antibodies are raised against recombinant proteins or against cells transfected with the target protein. If the target protein is highly glycosylated in its native form, evidence needs to be provided to show that antibodies derived in this way indeed detect the native glycoprotein.

Antibody dilution—why Is Determining the Dilution So Important?

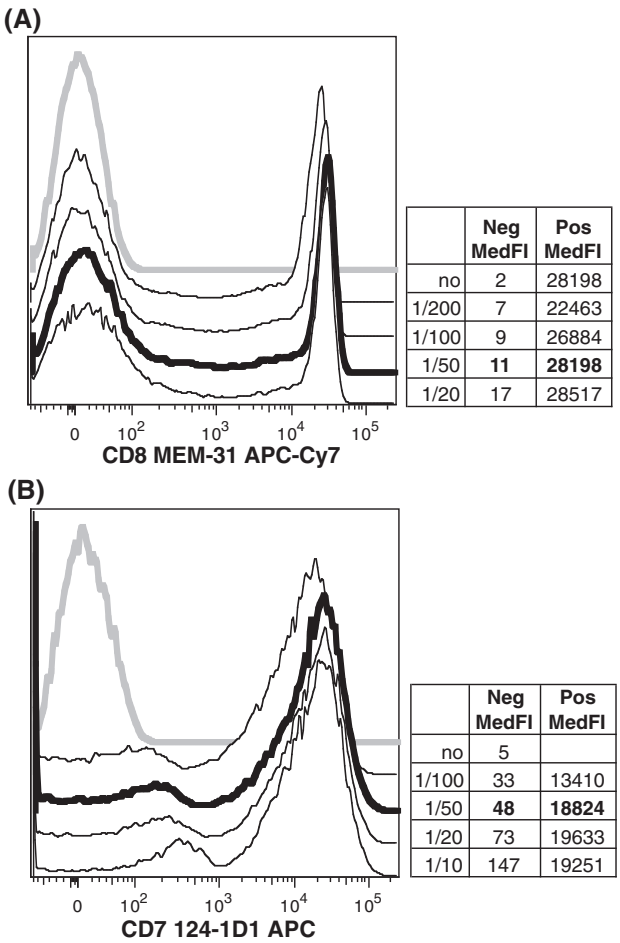
The determination of the proper dilution of antibodies is essential to ensure the specificity of the staining. The use of the improper dilution of the antibodies, which can generate unwanted background, is one of the major sources of poor quality results in flow cytometry. Moreover, using the recommended dilution of the antibody by the vendor is not always a guarantee of good performance under the specific conditions of our assay. To determine the optimal antibody concentration, staining with several dilutions of antibody must be performed (Fig. 2). The concentration that shows the best separation between negative versus positive cells and that exhibits negligible signal on non-target cells should be used (46). Frequently, the dilution would be lower than that recommended by the supplier with the additional benefit of spending less money. Titration should be performed with the sample and the number of cells that is to be used in our experiment. Antibodies with low affinity typically provide titration curves with no clear saturation plateau, and thus, are extremely prone to produce spurious, titer-dependent false-positive or false-negative results. A potential pitfall of antibodies with very high affinity is that they can be used at very low concentrations, making them prone to insufficient staining in a situation of antigen excess (47). In high concentrations, they can aggregate target cells (45,48).

Reproducibility and clonal identity—Can the Same Immunophenotyping Pattern Be Reproduced (with Other Clones or Other Batches of the Same Clones)?

Antibodies produced by different clones against a certain molecule can recognize distinct epitopes, present different affinities, or have several isotypes that can affect their staining performance. This is why it is so important that both companies, as well as users, detail the identity of the clones in their publications to guarantee the reproducibility of the results. If we are starting a new research or clinical study, it is advisable to test more than one clone to ensure that we can achieve reproducibility for our results. Thus, clone identification is a key point if we want to guarantee the robustness of the results generated by flow cytometry. Unfortunately, some companies only show the name of the reagent and not the clone name, or in some instances, even change the original clone name. Clones validates in HLDA workshops serve as benchmarking reference clones.

Lack of understanding and lack of commitment—How Do I Choose the Best Reagent and the Best Staining Conditions for my Experiment?

Although there is a significant effort in some antibody developers and antibody manufacturers to validate their products,



	Neg MedFI	Pos MedFI
no	2	28198
1/200	7	22463
1/100	9	26884
1/50	**11**	**28198**
1/20	17	28517

	Neg MedFI	Pos MedFI
no	5	
1/100	33	13410
1/50	**48**	**18824**
1/20	73	19633
1/10	147	19251

Figure 2. Antibody titration. (**A**) Lymphocytes: Titration of CD8 MEM-31 APC-Cy7 reagent. Median of negative cell remains unchanged and positive cells reach a plateau at 1/50 titer. (**B**) Lymphocytes: Titration of CD7 124-1D1 APC reagent. Median of negative cells increases with increasing titer, at 1/50 maximum median fluorescence is achieved for positive cells. This reagent will be prone to false-positive staining at higher than optimal titers.

there is also an urgent need to train researchers to understand the validation principles, to select reagents based on the validation data, and to use appropriate experimental conditions across the whole range of research applications. Thus, the research community, in collaboration with producers and vendors, should commit sufficient time, resources, and expertise to educate and train scientists (particularly junior researchers) in best practices for antibody-based experiments and their critical reviewing.

Antibody Validation Protocol

A validation protocol should provide solid evidence for the specificity of the antibody for the target antigen. Antibodies for flow cytometry should specifically be validated for this application, with a detailed sample preparation protocol and the cell sample to be analyzed (9,49). An antibody validated for another application, such as immunohistology or WB, is never guaranteed to perform well in flow cytometry. MAb validation for flow cytometry has its own peculiarities, and here, we provide a basic validation protocol to be used for this specific application. Summary of antibody information serves for the validation experiments' setup and should be accompanied validation file summarizing the data generated using this basic validation protocol (Fig. 3).

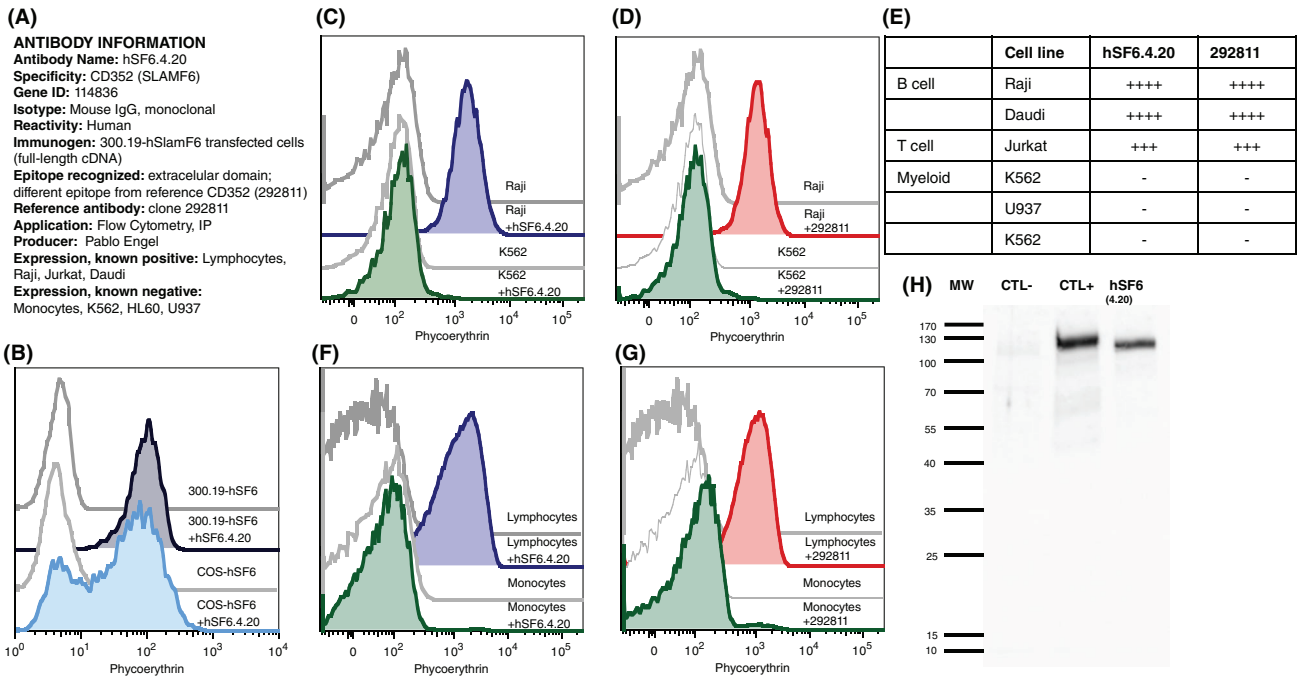
Transfectants Overexpressing the Target Molecule

The first step in the validation process consists of demonstrating the specificity of the antibody reactivity with cells that overexpress the target antigen using flow cytometry. Cells should be transfected with the cDNA encoding our target antigen (Fig. 3B). We must ensure that the untransfected cells do not express the target molecule. For example, monkey COS cells, the most popular cell line for transient transfections, express CD58 and CD109, which are recognized by most of the anti-human antibodies (50). Whenever possible, a well-validated antibody against the same protein has to be included in parallel. Alternatively, if no antibodies are available and to ensure that the target protein is expressed by the transfected cells, we can use epitope-tagged proteins, such as Green fluorescent protein (GFP) or hemagglutinin (HA).

As already described, it is also mandatory to titrate the antibodies to obtain the optimal dilution to enable sensitive detection while minimizing nonspecific background binding: It will be necessary to test the specificity of the reagent against other related proteins when the target antigen presents a high degree of homology with these other related proteins. Although these tests with transfected cells are a good indication of the binding to the target antigen, they are not sufficient to prove their specificity (see cross-reactivity issue above).

Downmodulation of the Expression

Several procedures that allow the downregulation of the target antigen can be used to strengthen the confidence in the specificity of the antibody. The comparison between the reactivity of the antibody with wild-type and KO deficient mice can be used. This is a powerful approach, but it has the limitation that it can only be used for antibodies against mouse proteins. Other limitations of this approach are the availability of cells from these mice. In some instances, the incomplete gene deletion of the target protein has proven to be a setback. in vitro approaches, such as siRNA protein downregulation, have been commonly used, but they pose their own challenges. Often, this technique is not easy to optimize, and the "off target" reduction of the expression is not an uncommon observation. This is especially relevant when we lack a validated antibody that we can use as a control. More recently, the generation of cell lines using CRISPR/Cas9 technology has been implemented by both companies and academic groups and has been used to validate antibodies (51,52). Several companies offer services to produce KO cell lines for specific target and others, such as Horizon Discovery, have generated already large panels of these cell lines. The problem is still to



	Cell line	hSF6.4.20	292811
B cell	Raji	++++	++++
	Daudi	++++	++++
T cell	Jurkat	+++	+++
Myeloid	K562	-	-
	U937	-	-
	K562	-	-

Figure 3. Antibody validation of anti-CD352 clone hSF6.4.20. (**A**) Antibody information. (**B**) Transfected cell lines COS (light blue) and 300.19 (dark blue) stained with clone hSF6.4.20 or secondary Ab only (gray). (**C**) Know positive cell line Raji (blue) and known negative cell line K562 (green) stained with clone hSF6.4.20 or secondary Ab only (gray). (**D**) The same lines (Raji in red, K562 in green) stained with reference clone 292,811 or secondary Ab only (gray). (**E**) Reactivity comparison with a reference clone 292,811. (**F**) Human leukocytes stained with clone hSF6.4.20 (Lymphocytes in blue, Monocytes in green) or secondary Ab only (gray). (**G**) Human leukocytes stained with reference clone 292,811 (red) or secondary Ab only (gray). (**H**) IP with clone hSF6.4.20 from biotin labeled 300.19-SLAMF6 transfected cells. Negative control (CTL-) anti-human Fc clone 29.5 and positive control (CTL+) anti-mouse Ly9 clone 7.144. [Color figure can be viewed at wileyonlinelibrary.com]

formally prove that they do not express the target antigen, for example, by PCR, since not all the cell lines are completely negative. In very rare cases, this strategy is not feasible, for example, when the protein is essential for the proliferation or the survival of the cells line. However, even with those limitations, genome editing will become a conventional strategy to improve antibody validation during the next years.

Recognition of Target Antigen on a Panel of Cell Lines

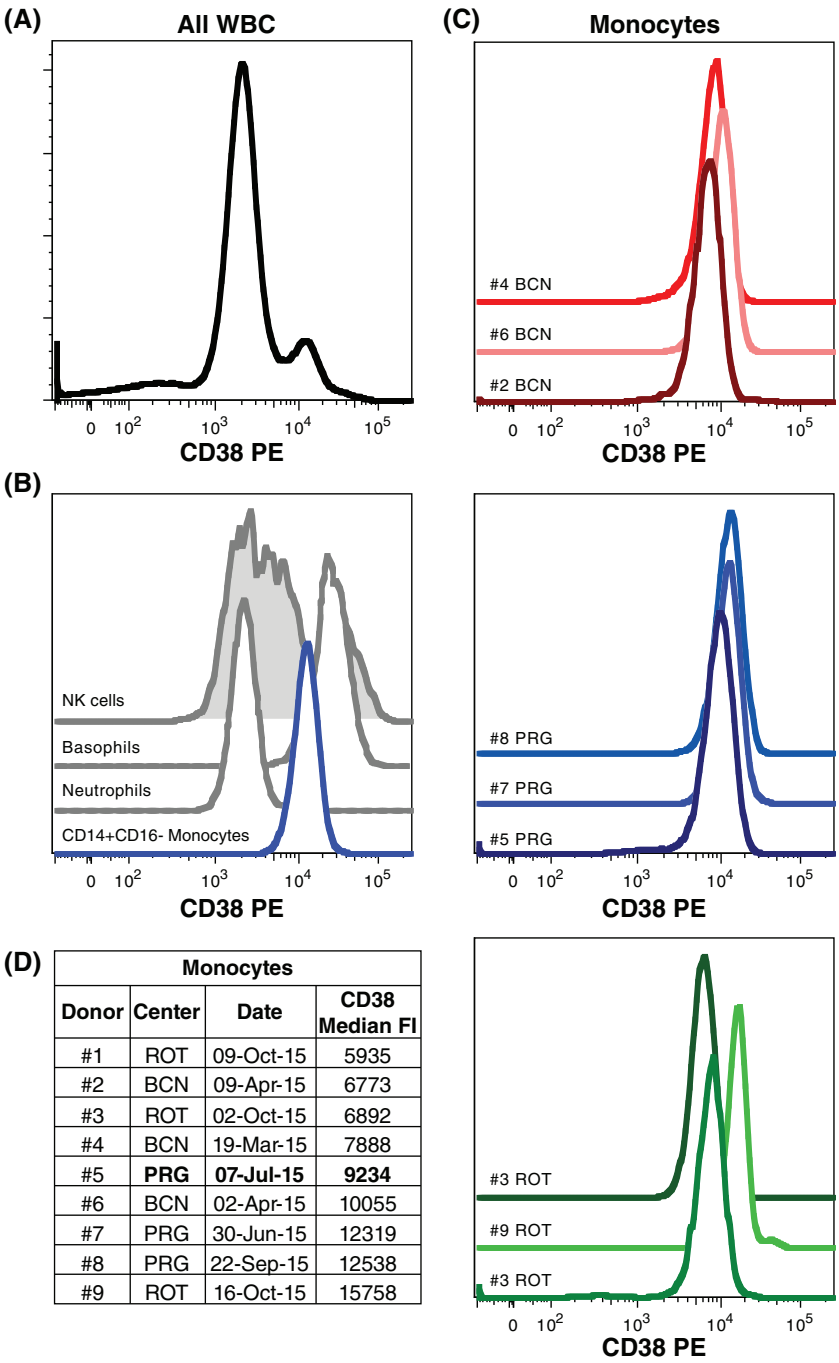
Cell lines that endogenously express or lack the protein of interest are widely used as positive and negative controls in antibody validation, especially for leukocyte cell surface molecules, because many cell lines, corresponding to most leukocyte populations and differentiation stages, are available. It is recommended to use at least two positive and two negative cell lines with confirmed positivity and negativity, respectively (Fig. 3C). Comparison of the staining profile to a reference clone is advisable (Fig. 3D,E). However, one limitation is that not all proteins are expressed on these cell lines.

Recognition of the Endogenous Protein and Expression Pattern

It is a relatively frequent finding that antibodies that are specific to the target antigen are not reactive with the endogenous or natural antigen. This is because of the current use of synthetic peptides, recombinant protein or transfected cells as a source for antigen immunization. Thus, proof of reactivity with the endogenous protein is key in the validation process (Fig. 3F). Disappointingly, some companies do not provide data showing reactivity with the natural target antigens. However, it should be mentioned that staining with the endogenous protein does not guarantee specific binding to the target antigen. For example, positive staining may be the product of cross reactivity with more than one molecule. In this case, the pattern of reactivity with different populations and subsets of cells can have an important value. It is important to choose the right sample to perform these studies. The knowledge of the expected expression pattern usually comes from publications and mRNA or proteomic databases. Ideally, a reference antibody should be used to ensure that our antibody expression pattern is identical to that observed with the reference antibody (Fig. 3G). In the absence of a well-validated reference antibody, these studies can be challenging.

Negative Controls Are Essential to Confirm Specificity

The negative controls are as important as the positive controls to confirm the specificity. Negative controls should consist of cells where your target protein is known to be absent. For example, the use of B cells (CD19+) to test T cell markers, such as CD3, CD4, or CD8, is necessary. However, it is often difficult to find cells that completely lack the expression of a certain target antigen. Even using knockout cell lines, we have to make sure that they are truly negative by testing the



Monocytes			
Donor	Center	Date	CD38 Median FI
#1	ROT	09-Oct-15	5935
#2	BCN	09-Apr-15	6773
#3	ROT	02-Oct-15	6892
#4	BCN	19-Mar-15	7888
#5	**PRG**	**07-Jul-15**	**9234**
#6	BCN	02-Apr-15	10055
#7	PRG	30-Jun-15	12319
#8	PRG	22-Sep-15	12538
#9	ROT	16-Oct-15	15758

Figure 4. CD38 clone HIT2 PE reproducibility of staining pattern of donors measured in Barcelona (BCN), Prague (PRG), and Rotterdam (ROT). (**A**) Single parameter staining profile can be complex on total WBC, (**B**) often owing to the particular intensity of different subsets that can have variable intensity such as NK cells. (**C**) Monocytes present with relatively stable inter-donor intensity (BCN donors in red, PRG in blue, ROT in green). (**D**) As a part of antibody conjugate characteristics, typical median expression of CD38 Fluorescence Intensity on Monocytes in standardized setup can be shown as median (bold) and minimum and maximum range, despite the fact that data were acquired on different donors, different instruments, and at different times. [Color figure can be viewed at wileyonlinelibrary.com]

mRNA levels using PCR or (ideally) mass spectrometry. In the case of pan-leukocytes markers (such as CD45), there is no negative control subset. In those cases negative cell lines or leukemic cells might be useful (46); however, care must be taken that autofluorescence is interpreted carefully comparing it with unstained sample.

Biochemical Validation

To complement the staining validation studies, it is also convenient to validate the antibodies with an alternative technique. Immunoprecipitation is one of the best options because it can be performed under experimental conditions similar to those used in the sample preparation method for

flow cytometry (Fig. 3H). Unlike flow cytometry alone, it will yield valuable information about the molecular mass of the target antigen and the isoforms recognized by the antibody. It may also unravel unwanted cross-reactivity with other proteins. Immunoprecipitation combined with mass spectrometry has recently proven to be a very powerful tool to validate antibody specificity. It allows the identification of proteins that selectively bind to an antibody, including any off-target proteins (53). The role of these high content techniques with a digital, and thus, data analysis amenable output will increase.

Recognition of the Same Target in Other Species

The reactivity and specificity for the target antigen should be validated for each specific species being studied. The observation of reactivity of a mAb with cells of another species does not guarantee that the antibody will recognize the orthologous antigen. Testing the expression patterns with different cells usually indicates whether the antibody recognizes the same protein in different species. Although in some cases, there may be a striking difference in the expression patterns between species. For example, CD2 is only expressed in T cells in humans, and in contrast, it is present in both T and B cells in rodents.

Manufacturing and Lot-to-Lot Reproducibility

One additional problem with conjugated antibodies in flow cytometry is the issue of lot-to-lot variability. The reasons can derive from differences in the manufacturing process, such as labeling procedures, but they can also be caused by improper conditions during the shipment or storage of the antibodies by the end user. Validation tests that control batch variability shall be performed and presented by the antibody manufacturer, but it is equally important to test any new lot with at least one positive and negative control and to perform the titration of the new antibody preparation by the end user. Whenever a flow cytometry test is performed as fully standardized (down to intensity level) or quantitative information is desired, signal intensity validation is necessary. When multiparameter flow cytometry is used, cell subsets serving as positive and negative controls can be gated within a sample and those can be used to control for staining intensity; this is true even when different donors are used over extended periods of time and in different laboratories if the same sample preparation protocol is used (Fig. 4). Description statistics (median, minimum to maximum) can be used for lot-to-lot variability characterization.

New Methods

Any new antibody validation methods that might have an impact on flow cytometry must be proven to be useful for cytometry reagents and must be amenable to the review process. The results shall be presented to the community in a concise manner in ideally, a single resource or via an aggregator of resources, and these should be updatable. This implies that those methods should be high throughput, reproducible and should produce a structured digital output. Ideally, a consensus approach that allows for benchmarking, side-by-side comparisons and even blind testing should be established and agreed upon. As suggested by Uhlen et al. (49) in the report of the International Working Group for Antibody Validation (IWGAV), a third pillar of antibody validation method can use a comparison of reactivity across many known cell types compared to that of a well-characterized "anchor" clone. This approach is particularly suitable for flow cytometry, where measurements of dozens, or even hundreds, of antibodies across multiple (dozens) subsets and/or characterized (cell-barcoded) cell lines are possible in a standardized, multi-laboratory fashion. Indeed, clustering patterns of two or more antibodies have been a principle of the CD workshops as discussed previously. With the employment of multicolor flow cytometry, more and better defined subsets can be resolved in parallel to the tested antibody in a relatively high-throughput fashion. HLDA has currently finished a pilot CD Maps study of 111 antibodies tested over 47 subsets in 12 donors in four laboratories. This data set will be used for benchmarking the antibody reactivity of additional clones to the same CD markers, and this approach will also be used for a new HLDA workshop planned for 2019. In the same time, the CD Maps data set will be released for public use at the hcdm.org website.

However, another high-throughput method for antibody validation was developed by Lund-Johansen's group, who used flow cytometry to quantify the immunoprecipitation of a target protein on a microbead (54), later adding the resolution of thousands of microbeads each immunoprecipitating its target protein from cell lysates after size-exclusion chromatography and differential detergent lysis (42,43,55). The addition of the automated analysis tool (56) and the creation of a pipeline for antibody reactivity profiling coupled to the mass-spectrometry validation (53) of target proteins has made this approach useful for the large-scale antibody performance validation of intracellular targets.

In conclusion, there are methods for antibody validation, performance benchmarking, and reactivity comparisons available; the next step is reaching a consensus that those shall be used, and perhaps more importantly, to find a viable model of vendor independent platform to build, maintain and supervise the resulting datasets produced by the community; however, all stakeholders need to support the building of this platform.

Surface, Cytoplasmic, and Nuclear Proteins and Phosphokinases as Targets of mAbs

Whereas the mAbs raised against the surface molecules used native proteins (or whole cells) as the immunization epitope, most of the antibodies against intracellular targets are typically being raised by genetically produced peptide immunization. In the course of sample preparation for flow cytometry, the cells must be fixed and permeabilized to make the target epitopes available for antibody binding. However, the procedure of fixation and permeabilization might alter the target epitope; thus, the applicability of those antibody reagents in

cytometry depends on whether the epitope is indeed present or lost, unobstructed and unaltered in the fixed target cell. A particular sample preparation (fixation and permeabilization) protocol is the key to successful staining for intracellular cytometry. Unfortunately, commercially available fixation and permeabilization buffers are typically supplied without information about their composition, so their performance with a particular target protein—detection antibody conjugate must be evaluated side-by-side to select the best compromise solution effective for the simultaneous detection of several intracellular targets as exemplified by Papagno et al. (57) for intracellular cytokine staining and by Law et al.(58) for FoxP3 staining. It was shown for phospho-protein detection that fixation by 2% or 4% formaldehyde followed by methanol epitope unmasking (50–90%) is correlated with the better signal of the phospho-protein at the cost of decreased signal for surface anti-CD3 staining (30). For further reading, refer to chapters IV.6. and VII.15 in the study by Cossariza et al. (59).

Some information about the sensitivity of the antibody epitope to fixation or conjugate performance with a given fixation protocol can be found on the particular manufacturers' website, but this is unfortunately neither citable (with DOI) nor aggregated over several sources.

In summary, antibody conjugate malfunction can be caused by a suboptimal protocol chosen for cell fixation and permeabilization. The challenge of current multidimensional cytometry is to compromise on a single protocol that enables the detection of all intended targets with a single fixation method, even if that fixation method may not actually be the ideal for each clone individually. For antibodies that currently require unique fixation and permeabilization protocols, new clones should be endeavored to be created using immunogens most likely to produce antibodies compatible with this single fixation method. While the clone is still in development, manufacturers should test and subsequently communicate that information about clone performance under multiple fixation and permeabilization conditions.

How Do we Increase Trust in Antibody Reagents?

To increase confidence in the quality of antibodies, we must first delineate the responsibility of the manufacturer of the antibody-fluorophore conjugates and the responsibility of its users. As with all technological advancements in this arena, novel antigen identification and classification have been paralleled by improvements in process and the quality of antibody products over the last 30 years (see general commentary about promises for future antibody validation by Baker (50)). What has remained limited is the access to the information of how that clone was developed, validated, and manufactured by the end user. The sort of information manufacturers thought relevant to provide in the past was limited to what could fit in the limited space of product sheets included with an antibody. Typically, the information provided to the end user was not the only validation conducted on that antibody. With the advent of the digital revolution, we expect the ability to interface with such large amounts of data as a consumer right.

Transparency is the most important factor in building confidence in reagents and beginning to weed out those clones that are not up to a high standard of performance from distributed protocols and publication. However, that responsibility is a shared one. Independent validation either by the replication of positive or negative results akin to that provided by the manufacturer or validation with additional tissues or disease states to further expand our understanding of the clone further solidifies our trust in the quality of the clone. Efforts, such as HLDA or other collaborative efforts, and databases are excellent examples (35). Nonetheless, quantitative antibody performance benchmarking in a standardized and reproducible manner will provide basis for informed reagent selection. Human Cell Differentiation Molecule (HCDM) council is organizing the next HLDA11 workshop with the inclusion of benchmarking and quantitative comparisons. Likewise, the CDMaps project will enable benchmarking of reagents. Even with all that effort, some poorly performing reagents will contaminate the literature and the market. For example, antibodies that were tested within HLDA workshops may have different affinity or may fail in particular applications (44).

Additionally, just as manufacturer results should be more transparent, there should also be a higher standard as to the data that is shared with the community. First and foremost, each use of a mAb in the scientific literature should use an unequivocal identifier (typically a clone name, fluorochrome, manufacturer and product code) (60); it is the responsibility of the authors to provide this information just as it is the responsibility of the reviewers to enforce this requirement. When complete, this information is searchable by engines like CiteAb (61) or Antibodypedia (62). Negative results should be shared along with the positive results in publications, and flow cytometry data should be deposited in repositories (FlowRepository (63) or ImmPort (64)) in compliance with MIFlowCyt requirements (65); this is necessary so that the data described in each paper are available to the community to be transparent on gating schemes and interassay reproducibility. Ideally, such publicly available data should be referenced by the vendors. How many "irreproducible" results might have been caught by the community if there were more thorough reviews of the methods? These are the responsibilities of the end user of antibodies: transparency and the understanding of their detailed protocols, applications, and analytical methods. Process transparency on all sides will increase confidence in the antibody in the end.

Concluding Remarks

Antibody reagent conjugates are key components in the current cytometry analyses. The reproducibility potential of cytometry is great as is the potential for large data analysis and for data mining. To fully use the cytometry potential, antibody conjugates must perform flawlessly, and thus, mechanisms for the validation of their performance must be in place and must be further developed to increase trust in the

reagents that work well and to remove antibody reagents that failed those expectations after widespread use. Antibody reagents act as cytometry tools and have challenges in development, validation, and proper use. A concerted extra effort of antibody developers, manufacturers, users, and publishers is essential for the proper usage of antibody reagents in cytometry in the future.

Users should be knowledgeable about antibody validation principles (9,41,49,66), they should choose antibody reagents carefully, based on the amount and quality of validation data presented by the developers and manufacturers. Users should present their own validation data of their method, including negative results of the direct comparisons of clones, reagents, and sample preparation protocols. As a positive good practice example, we should encourage the publication of Optimized Multicolor Immunofluorescence Panels format in *Cytometry A* journal (67,68). Users shall use the MiFlowCyt requirements (65,69) checklist to provide complete experimental information and should deposit the data into a repository for reanalysis and reproducibility check.

The developers of antibody reagents should perform multimodal validation to address all the issues of specificity, cross-reactivity, epitope nature, antibody dilution and clone identity (see section Antibody validation protocol). They should present the validation data in full detail so that those can be reproduced. Manufacturers should preserve the clones' identities, make all data validation data available, including sample preparation protocols, staining tests on positive and negative cells of interest, titrations and should also share the negative results obtained using suboptimal protocols (especially in assays using fixation and permeabilization).

Publishers, editors, and reviewers should demand that the experimental procedures details including clone identities are provided. Essentially, it is important that a MiFlowCyt adherence is controlled and enforced.

All the stakeholders should work together to build structure and capacities for making available the necessary data about antibody reagents that would favor the choices of reagents based on the validation data rather than random picks that potentially litter the scientific literature with irreproducible conclusions. The structured sharing of validation data should be amenable to the aggregation of relevant validation information and should guide the user to select quality reagents. HLDA and CD workshops show successful examples of coordinated and community-driven efforts to bring order to the chaos of cell surface molecules targeting antibodies (35,70). EuroMabNet has focused on immunohistochemistry reagents (9). Currently, HLDA is building a CDMaps resource that will interface the expression information about CD marker expression. The CD Map information can be used in validation experiments as well as it can allow for benchmarking antibody reagents.

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