Received: 6 January 2023 | Revised: 30 September 2023 | Accepted: 29 November 2023

DOI: 10.1002/cyto.b.22155

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Standardization of flow cytometric detection of antigen expression

Linhua Tian[1] | Aaron R. Nelson[2] | Tyler Lowe[2] | Linda Weaver[2] | Constance Yuan[2] | Hao-Wei Wang[2] | Paul DeRose[1] | Maryalice Stetler-Stevenson[2] | Lili Wang[1]

[1]Biosystems and Biomaterials Division, National Institute of Standards and Technology (NIST), Gaithersburg, Maryland, USA

[2]Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Correspondence
Dr. Maryalice Stetler-Stevenson, Laboratory of Pathology, NCI, NIH, Building 10, Room 3S235, Bethesda, MD, 20892, USA.
Email: mstetstev@comcast.net

Dr. Lili Wang, Biosystems and Biomaterials Division, NIST, 100 Bureau Drive, Gaithersburg, MD 20899, USA.
Email: lili.wang@nist.gov

Abstract

Since response to antigen-based immunotherapy relies upon the level of tumor antigen expression we developed an antigen quantification assay using ABC values. Antigen quantification as a clinical assay requires methods for quality control and for interlaboratory and inter-cytometer platform standardization. A single lot of Cytotrol™ Lyophilized Control Cells (Beckman Coulter) used for all studies. The variability in antigen quantification across 4 different instrument platforms in 2 separate laboratories was evaluated. The effect of the antibody clone utilized, importance of custom 1:1 molar ratio (fluorophore to protein, F/P) verses off-the-shelf antibodies, and QuantiBrite PE calibration verses linearity calibration combined with a single point scale transformation with CD4 as reference were determined. Use of single lot control cells allowed validation of reproducibility between flow cytometer platforms and laboratories and allowed assessment of different antibody lots, cocktail preparation, and different antibody clones. Off the shelf antibody preparations provide reproducible estimates of antigen density, however custom 1:1 unimolar antibody preparations should be utilized for definitive measurement of antigen expression.Geometric Mean fluorescent Intensity (GeoMFI) was not comparable across instruments and inter-laboratory. The use of CD4 as the reference marker can minimize variability in ABC values. Comparable antigen quantification is vital in managing patients receiving antigen-based immunotherapy. If this assay is to be utilized in a clinical setting, quality control methods have to be instituted to assure reproducibility and allow validation across laboratories. We have demonstrated that use of a lyophilized cell control is highly valuable in achieveing these goals.

KEYWORDS
acute leukemia, Flow cytometry, Leukemia, Qunatification, Standardization

1 | INTRODUCTION

Immunotherapies, such as bispecific T-Cell engagers (BiTE) or chimeric antigen receptor (CAR) T-cell therapy, have radically changed therapy in cancer patients with relapsed or chemotherapy refractory disease and their possible incorporation into standard therapy is being investigated (Lee et al., 2015; Topp et al., 2015). Surface expression of the targeted antigen is required for CAR T-cell binding and killing;

the level of antigen expression can impact treatment response and duration. At lower target antigen densities, CAR T-cells are less potent and produce decreased cytokine levels, such as IFN-gamma or IL-2, despite augmentation with co-stimulatory molecules or alterations of CAR T-cell affinity binding (Chmielewski et al., 2011). The finding that CD22 dim disease is prone for relapse after CD22 CAR therapy (Fry et al., 2018) and that dim disease limits anti-tumor efficacy (Ramakrishna et al., 2019) indicates the importance of sufficient antigen density for CAR T-cell efficacy. In a preclinical model of CAR T-cells targeting anaplastic lymphoma kinase (ALK), lower antigen expression also limited the anti-tumor efficacy of the CAR (Walker et al., 2017). In addition, tumor cell evolution resulting in target antigen modulation (the decreased level of expression or antigen loss) is emerging as a mechanism of resistance (Majzner & Mackall, 2018; Shalabi et al., 2018; Sotillo et al., 2015; Yu et al., 2017).

Antigen density prior to immunotherapy is receiving attention and may impact feasibility of future antigen targeted therapies (Libert et al., 2020; Walker et al., 2017). Furthermore, antigen-dim disease will likely become a more frequent occurrence with the rise of sequential immunotherapies. In a study, the majority of patients with relapsed/refractory disease (B-lymphoblastic leukemia-B-ALL, diffuse large B-cell lymphoma-DLBCL) post CD19 targeted therapy, had CD19 negative, CD19 partial, or CD19 dim disease. This impacted the efficacy of additional immunotherapies (Libert et al., 2020). Some patients showed dynamic changes in surface CD19 expression, warranting the serial evaluation of surface CD19 expression to monitor for renewed susceptibility to CD19 targeting (Libert et al., 2020). In anti-CD22 targeting therapy for B-ALL, prior targeting with anti-CD22 Inotuzumab can result in a lower CD22 expression (Bhojwani et al., 2019; Yates et al., 2018), which leads to decreased subsequent CD22 CAR T-cell activity (Fry et al., 2018).

Additionally, targeting one antigen may impact the level of expression of another antigen, reducing efficacy of immune-based therapies. CD19 and CD22 expression levels trend together (Libert et al., 2020). With CD19 loss post-therapy, there is a corresponding decrease in CD22 which may impact response to subsequent therapeutic strategies. Furthermore, in immunotherapy-naïve patients, low CD19 expression had a correlation with low CD22 expression (Libert et al., 2020).

Due to the inherent variability in qualitative estimates of antigen density (e.g. 1+, 2+, etc. or dim, moderate, bright), there is an increasing interest in flow cytometric quantification of cell surface expression of antigens targeted by immunotherapies. Geometric Mean Fluorescent Intensity (GeoMFI) has been utilized in studies in an effort to provide more accurate quantification, however GeoMFI may not be reproducible across different instruments and laboratories. Antibodies bound per cell (ABC) is a unit of measurement for biomarker expression that is instrument independent (Davis et al., 1998). In the well-known BDIS QuantiBrite™ method, standard beads with known quantities of fluorescent phycoerythrin (PE) are run and hence a standard curve of number of PE molecules versus fluorescence intensities of the beads is constructed. This fluorescent calibration curve allows measured fluorescence signals given by cells to be converted to the ABC values as expression levels of cell surface antigens. This technique can be applied to the study of hematolymphoid neoplastic cells (Salem et al., 2018; Tembhare et al., 2013). This expression analysis method is straightforward; however, result comparability across different instrument platforms, reagent lots, operators, and laboratories has not yet been demonstrated. Assessing the assay variability of flow cytometric quantitation, including interinstrument, intralaboratory, interlaboratory, and antibody lot-to-lot variability, is therefore critical in developing assay standardization to enable broad clinical utility and impact. To study potential sources of assay variability, the levels of CD4, CD19, CD20, and CD22 expression were investigated using Beckman-Coulter's Cytotrol™ control cells, a known stable and uniform lyophilized PBMC control, across instruments, laboratories, and antibody lots. Because the CD4 expression was measured in this study, we compared an alternative quantification scheme based on the use of CD4 as a reference marker (Degheidy et al., 2016; Wang et al., 2012, 2014, 2016) to the QuantiBrite™ PE calibration method.

2 | METHODS

Cell processing and data acquisition: To keep the specimen identical across experiments, a single lot of Cytotrol™ Lyophilized Control Cells (Beckman Coulter) was used for all studies. Cells were reconstituted at a concentration of $\sim 1.0 \times 10^6$ per mL per manufacturers recommendations. A 100 µL aliquot of Cytotrol™ cells was incubated with one of the following single antibodies: CD4PE (PE) clone 13B8.2 Beckman-Coulter (BC); CD4PE clone SK3 Becton Dickinson Biosciences (BD); CD19PE clone J3-119 BC; CD19PE clone SJ25C1 BD; CD20PE clone B9E9 BC; CD20PE clone L27 BD; CD22PE clone SJ10.1H11 BC; CD22PE clone S-HCL-1 BD or an antibody cocktail with CD45v500 clone H130 BD added to the single antibodies per manufacturers' recommendations and as previously reported (Lee et al., 2015). Briefly, Cytotrol™ cells were incubated at room temperature in the dark for 30 min, washed with PBA (PBS, 0.5% BSA, 0.05% sodium azide) and resuspended in PBA, and stored at 4°C in the dark before acquisition within 8 h of staining. Flow cytometers were cleaned prior to acquisition by running 10% bleach solution on the cytometer sample probe for 5 min followed by 5 min deionized water. Specimens were acquired on the following flow cytometric instruments: two BD FACSCanto II and one Lyric instruments in Laboratory 1; one Attune NxT and one CytoFlex LX instruments in Laboratory 2. QuantiBrite™ standard beads (BD Biosciences) were run the same day on each instrument. Data was analyzed using FSCExpress (De Novo Software) and QuantiCalc (BD Biosciences) software to generate standard curve with QuantiBrite™ beads and the ABC values determined. This directly provides accurate antigen quantitation in antibodies with a 1:1 molar ratio of PE to antibody (unimolar). Due to insufficient availability of unimolar PE-antibody conjugates, non-unimolar antibodies must be used in some assays. As the fluorescence to protein (F/P) ratio of antibodies may vary in non-unimolar antibodies, the value of adjusting the ABC value using the known (F/P) ratio provided by the manufacturers as previously described

(Tembhare et al., 2013) was investigated by comparing QunatiBrite generated ABC values with and without adjustment of F/P ratio.

Instrument comparison: Potential variability in antigen quantitation across platforms and laboratories was assessed by simultaneous evaluation of identical specimens in multiple platforms and in two laboratories. In order to evaluate intralaboratory instrument variation in ABC values, results were compared across two identical flow cytometer platforms, namely two FACSCanto II instruments, in the same laboratory by running triplicate identical specimens prepared and acquired on the same day. To compare cross platform variations in PE fluorescence channels due to instrument filter differences and to explore possible interlaboratory variation identical specimens were run on 2 BD FACSCanto II instruments and 1 BD Lyric instrument in laboratory 1 as well as an Attune and CytoFlex instrument in laboratory 2 on the same day.

Evaluation of the effect of antibody clone and quality on results: To evaluate the effect of the antibody clone utilized in antigen quantitation, two different anti-CD4, anti-CD19, anti-CD20, and anti-CD22 antibody clones were evaluated in identical specimens across 4 flow cytometer platforms (2 FACSCanto II, 1 Attune NxT, and 1 CytoFlex LX) in two laboratories. To verify the importance of a custom antibody preparation of a 1:1 molar ratio (fluorophore to protein, F/P), a commercial off-the-shelf 1:1 (fluorophore to protein) CD20 antibody was compared to the unimolar CD20 antibody conjugate from the same manufacturer across a FACSCanto II and Lyric instruments in Laboratory 1 and Attune NxT and Cytoflex instruments in Laboratory 2.

Evaluation of potential technical variability due to operator: Experiments using the identical antibody clone and lot number were performed on two separate days and acquired on the same 2 intralaboratory instrument platforms, namely two BDIS FACSCanto II in Laboratory 1.

Comparison of two antigen quantification methods: Intralaboratory variability on CD20 quantification was evaluated using commercial antibodies CD4 and CD20, two cytometer platforms (Attune NxT and CytoFlex LX), and two quantification schemes, namely QuantiBrite PE calibration and a linearity calibration combined with a single point scale transformation with CD4 as the reference marker (Degheidy et al., 2016; Wang et al., 2016).

3 | RESULTS

Cytotrol™ cells were utilized to evaluate possible variables affecting ABC values and to determine their usefulness as a possible QC reagent in developing and monitoring ABC testing. In order to evaluate the use of Cytotrol™ cells as a QC reagent for instrument comparison, triplicate Cytotrol™ specimens stained with the same lot of CD19 PE and CD22 PE were run on two separate BDIS FACSCanto II instruments on the same day. Use of control cells provided an excellent tool to evaluate intra- and inter-instrument reproducibility of ABC results (Table 1). As reproducibility of results over time is critical in a clinical laboratory, a repeat test was performed on both instruments 3 months later, showing good consistency of the measurements. The results demonstrated the utility of control cells to evaluate the longitudinal reproducibility of ABC values (Table 2).

TABLE 1 Comparison of CD19 and CD22 ABC values determined on two FACSCanto II instruments in same laboratory.

	CD19PE	CD22PE
	Clone SJ25C1	Clone S-HCL-1
BDIS FACSCanto A		
Mean ABC Values	7020	9329
SD	11	108
CV	0.**0016**	0.0116
BDIS FACSCanto B		
Mean ABC Values	7114	9505
SD	58	136
CV	0.0082	0.0143

Note: The mean, standard deviation (SD), and coefficient of variation (CV) from triplicate Cytotrol™ cells stained with CD19PE, Clone SJ25C1 from Beckman-Coulter and CD22PE, Clone S-HCL-1 from BD Biosciences.

Clinical laboratories are required to perform quality control on all new antibody lots to determine if the quality is the same as previous lots. This is typically accomplished by comparison of the newly purchased antibody lot to the previous antibody lot. Problems occur when a laboratory depletes the previous antibody lot before receipt of the new one or the old lot exceeds the expiration date. To assess the utility of control cells to perform lot testing, Cytotrol™ cells were stained in triplicate with two different lots of CD19, CD20, and CD22 and run on an Attune NxT (Table 3). The difference in the two CD20PE lots was larger than that observed with the other antibodies. This may be due to the greater difference in the F/P ratio for CD20 (difference in the F/P ratio of lots: 0.043 (CD19), 0.213 (CD20), 0.08 (CD22), indicating that this may be an important variable in ABC determinations. Adjusting the CD20 values for F:P ratio (formula: ABC/(F/P ratio) = adjusted value) did not correct the difference in ABC values between the two CD20 lots. This may be due to a number of causes, including an inherent problem in a specific lot of antibody, degradation of one antibody lot over time, or that a significant difference in the F/P ratio of an antibody indicates a disparity that cannot be simply corrected by the F/P ratio adjustment. The results demonstrate the utility of control cells in antibody lot testing.

The utility of control cells in Quality Control of cocktail preparation was also evaluated. Triplicate Cytotrol™ specimens stained with two cocktails containing CD4 PE, clone SK3 and CD45 v500, clone H130 from identical lots and prepared by two different individuals in two separate laboratories were acquired on the same Attune NxT cytometer and CD4 PE ABC values determined. The results are CD4PE ABC mean 26,061 (SD 77) for Cocktail preparation 1 and CD4PE ABC mean 26,271 (SD 259) for Cocktail preparation 2, respectively, demonstrating result comparability across cocktail preparations can be easily obtained.

Antibody clone selection is important as antibody clones binding to different antigen epitopes might result in variation in ABC values.

TABLE 2 Evaluation of ABC values at two different time points testing longitudinal reproducibility.

	CD19PE Clone S125C1 Time zero	CD19PE Clone S125C1 Time point 2	CD22PE Clone S-HCL-1 Time zero	CD22PE Clone S-HCL-1 Time point 2
BDIS FACSCanto A	7020	7714	9329	8945
BDIS FACSCanto B	7114	7504	9505	9244

Note: Mean ABC values from Cytotrol™ cells stained with CD19PE, Clone SJ25C1 from Beckman-Coulter and CD22PE, Clone S-HCL-1 from BD Biosciences. Time Zero is the mean value from triplicate determinations (Table 1) and is used for future testing evaluation. Time Point 2 is a single determination 3 months later indicating longitudinal reproducibility.

TABLE 3 Comparison of ABC values obtained with two different antibody lots.

Antibody (lot)	ABC value (values adjusted for F/P)
CD19PE (9151856): F/P 1.018	
Mean	3854 (3785)
SD	163
CV	0.0423
CD19PE (9066849): F/P 0.975	
Mean	4073 (4177)
SD	83
CV	0.0204
CD20 PE (9176021): F/P 0.897	
Mean	14,597 (16,273)
SD	282
CV	0.0193
CD20 PE (9044581): F/P 1.11	
Mean	13,001 (11,712)
SD	749
CV	0.0576
CD22 PE (9207363): F/P 1.03	
Mean	7114 (6906)
SD	64
CV	0.0090
CD22 PE (9038654) F/P 1.11	
Mean	6908 (6224)
SD	61
CV	0.0088

Note: The mean, standard deviation (SD), and coefficient of variation (CV) from triplicate Cytotrol™ cells stained with 2 different lots of CD19PE (clone SJ25C1), CD20PE (clone L27), and CD22PE (clone S-HCL-1) from BD Biosciences. Specimens run on the same day using the same Attune NxT cytometer.

Triplicate Cytotrol™ specimens stained with CD19 PE (clone SJ25C1), CD19 PE (clone J3-119), CD20 PE (clone L27), CD20 PE (clone B9E9), CD22 PE (clone S-HCL-1), CD22 PE (clone SJ10.1H11), CD4 PE (clone SK3), and CD4 PE (clone 13B8.2) from BD Biosciences and Beckman Coulter, respectively, were run a BDIS FACSCanto instrument on the same day. As shown in Table 4, the use of control cells allowed the assessment of comparability of antibody clones targeting the same antigen. In all cases there were differences in ABC values with the highest difference observed between the CD20 clones. Again, adjusting the ABC values for the F/P ratio did not correct the difference in ABC values. Final testing should be validated on live cells as observed differences may be secondary to the effect of lyophilization on the epitopes targeted.

Flow cytometer platforms differ across laboratories introducing a possible source of variability in ABC values. Four different flow cytometer platforms were used, including BD FACSCanto II, BD FACS Lyric, Thermo Fisher's Attune NxT, and Beckman-Coulter's CytoFlex LX cytometers, with which optical configurations of the PE channel are different, that is, under different laser excitations, 488 or 561 nm and with different bandpass filters such as 585 ± 21 nm and 585 ± 8 nm. In order to utilize the QuantiBrite PE calibration scheme across different instrument platforms, the shapes of PE fluorescence spectra from PBMC stained with PE-labeled antibodies and QuantiBrite PE beads have to be closely matched at the wavelength range defined by the bandpass filters. Figure 1 shows four reasonably matched PE fluorescence spectra obtained from PBMC stained with PE-labeled antibodies and QuantiBrite PE beads within the wavelength range of 585 ± 21 nm under either 488 nm or 561 nm laser excitation. Higher fluorescence background signals for QuantiBrite PE beads with regard to those for PE-labeled PBMC were observed under 488 nm laser excitation due to unavailability of unlabeled beads (QuantiBrite beads without PE labels) for proper background subtraction. The two pairs of fluorescence spectra under the two laser excitations match reasonably well within the wavelength range defined by bandpass filter of 585 ± 21 nm. These results indicate that the ABC values determined based on QuantiBrite PE calibration can be independent of the cytometer platforms used.

We next compared ABC values obtained using Cytotrol™ control cells stained with cocktails prepared separately in the two individual laboratories containing the same lots of CD4 PE, CD19 PE, and CD22, and run on the four cytometers in the two laboratories. As shown in Table 5, control cells allow comparison of quantification across different flow cytometer platforms and laboratories. ABC values obtained are consistent across instrument platforms as expected and with cocktail preparation by two different laboratorians using the same reagents. This indicates the use of control cells can evaluate and

TABLE 4 Comparison of different antibody clones in ABC value determination.

Conjugate/Mfr.	ABC value (F/P adjusted)
CD19 BC clone J3-119: F/P 1.19	
Mean	9941 (8354)
SD	95
CV	0.0096
CD19 BDIS clone SJ25C1: F/P 1.018	
Mean	6789 (6669)
SD	185
CV	0.0272
CD20 BC clone B9E9: F/P 1.0	
Mean	12,398 (12,398)
SD	430
CV	0.0346
CD20 BDIS clone L27: F/P 0.897	
Mean	19,480 (21,717)
SD	397
CV	0.0204
CD22 BC clone SJ10.1H11: F/P 0.8	
Mean	5066 (6333)
SD	69
CV	0.0136
CD22 BDIS clone S-HCL-1: F/P 1.03	
Mean	9710 (9427)
SD	14
CV	0.0014
CD4 BC clone 13B8.2: F/P 1.2	
Mean	33,715 (28,096)
SD	340
CV	0.0101
CD4 BDIS clone SK3: F/P 1.094	
Mean	29,721 (27,167)
SD	160
CV	0.0054

Note: The mean, standard deviation (SD), and coefficient of variation (CV) from triplicate Cytotrol™ cells stained with different antibody clones from BC-Beckman Coulter and BDIS-BD Bioscences and run on a BDIS FACSCanto.

validate interlaboratory agreement in determining ABC values across multiple platforms.

To minimize the variabilities due to antibody lot difference, cocktail preparation, and sample staining on the determination of ABC values and focus soley on instrument variability, Cytotrol™ cells were stained in triplicate with a cocktail containing CD4 PE, clone SK3 and CD45 v500, clone H130 (BDIS) prepared by a single individual using the same antibody lots on all specimens. The stained cells were acquired on a FACSCanto II and a Lyric in Laboratory 1 and an Attune and a CytoFlex in Laboratory 2. CD45 staining was utilized to gate out debris. The data shown in Table 6 demonstrate a large variation in GeoMFI across instruments making the comparison of GeoMFI across cytometers not useful, however, the ABC values based on QuantiBrite PE calibration were consistent across instruments.

For definitive quantification of the actual number of antigens expressed on cell surface, antibodies with a 1:1 molar ratio of PE to antibody (unimolar) should be used. Unfortunately, very few unimolar antibodies are available and nonunimolar antibodies must be used. Studies of ABC values using nonunimolar antibodies have, however, resulted in clinically useful prognostic data across serial patient specimens collected over time periods ranging from months to years and using different antibody lots with F/P ratios that were close to 1.0 (Bhojwani et al., 2019; Libert et al., 2020). We undertook a comparison of unimolar antibody to commercial, off-the-shelf antibody with an F/P ratio close to 1. Cytotrol™ cells were stained in triplicate with either a cocktail containing off-the-shelf CD20 PE, clone L27 and CD45 v500, clone H130 or a second cocktail with unimolar CD20 PE, clone L27 and CD45 v500, clone H130 (BD Bioscences), prepared by the same individual using the same antibody lots on all specimens. The stained cells were acquired on a FACSCanto II, a Lyric (Laboratory 1), an Attune, and a CytoFlex (Laboratory 2). The mean GeoMFI values and associated SDs of CD20 obtained are given for the off-the-shelf CD20 PE and the unimolar CD20 PE in Table 7. Large variation in GeoMFI values were observed across instrument platforms; however, the ABC values based on QuantiBrite PE calibration were very consistent for the use of unimolar CD20 PE and reasonably comparable for the off-the-shelf CD20 PE across instruments (Table 7). Additionally, CD20 ABC values were higher with unimolar verses off-the-shelf CD20 PE antibody, despite the off-the-shelf CD20 PE antibody having a F/P ratio close to 1. Adjusting the ABC value using the known (F/P) ratio provided by the manufacturers did not result in agreement between the unimolar and off the shelf antibodies. This is interpreted as intrinsic differences in the antibody preparation with the unimolar antibody undergoing further purification. An antibody with a F/P ratio of close to 1 still contains antibodies without fluorophore label, labeled with a single PE, or labeled with ≥2PE molecules, and that unlabeled antibody has a much higher binding affinity to antigen, resulting in lower GeoMFI and ABC values in the off-the shelf antibody.

We further explore the use of CD4 as a reference marker for quantifying CD20 expression levels (Degheidy et al., 2016; Wang et al., 2016). Corrected CD20 ABC values can be calculated by using the following equation:

$$\mathrm{ABC}_{\mathrm{CD20}} = \frac{\mathrm{ABC}_{\mathrm{CD20}}}{\mathrm{ABC}_{\mathrm{CD4}}} \times 40{,}000, \quad (1)$$

where the value of 40,000 is a known value of CD4 ABC for fixed whole blood and Cytotrol control cells using the off-the-shelf CD4 PE clone SK3 reagent (Davis et al., 1998; Wang et al., 2012, 2014). The resulted CD20 ABC values with CD4 measured on the same cytometer serving as the reference marker are provided in the last column of Table 7 for the use of the off-the-shelf CD20 PE and unimolar CD20 PE. Both ABC values of CD20 and CD4 on the right side of the

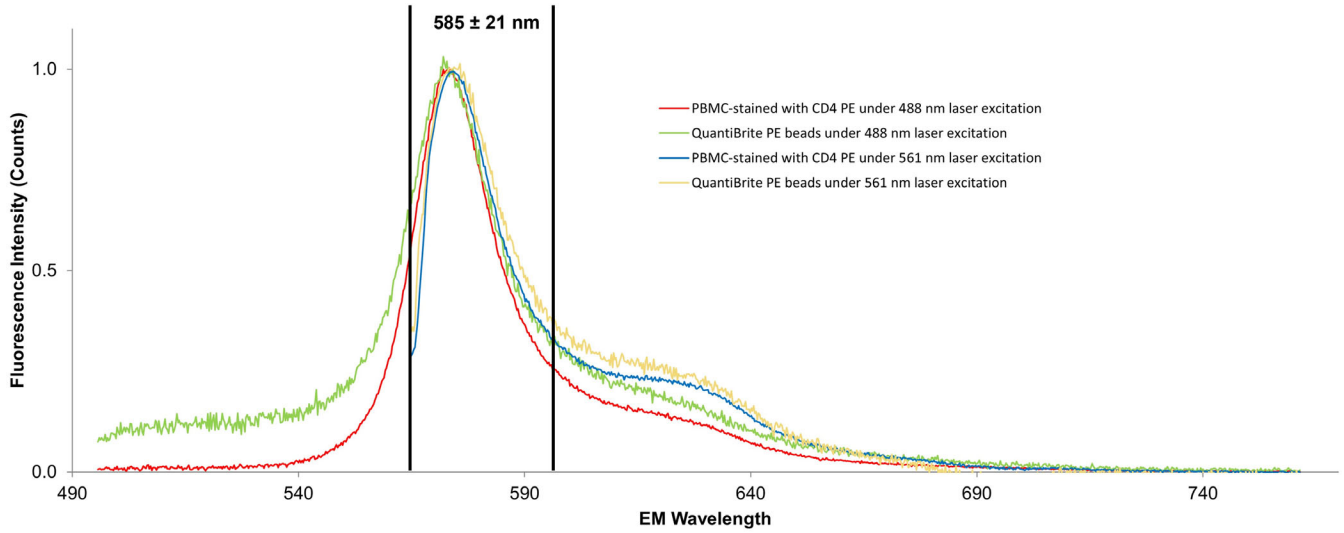

FIGURE 1 Normalized PE fluorescence spectra of PBMC stained with CD4 PE (clone SK3) and QuantiBrite PE beads in PBS, pH 7.4 under either 488 or 561 nm laser excitation using a calibrated CCD-based fluorimeter (DeRose et al., 2020). The fluorescence spectra of CD4 PE-stained PBMC were corrected by subtracting the background signal from unstained PBMC; however, for QuantBrite PE beads, background subtraction was made using PBS only. The wavelength range enclosed by the two black vertical lines is defined by the bandwidth filter of 585 ± 21 nm. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Comparison of different flow cytometer platforms in ABC determination.

Platform	CD4 ABC	CD19 ABC	CD22 ABC
Canto A			
Mean	33,715	9942	5066
SD	408	113	55
CV	0.0121	0.0114	0.0109
Canto B			
Mean	34,353	11,163	5133
SD	481	159	12
CV	0.0140	0.0142	0.0023
Attune NxT			
Mean	32,297	9712	3932
SD	271	162	14
CV	0.0084	0.0167	0.0036
CytoFlex LX			
Mean	36,422	9116	4843
SD	215	147	94
CV	0.0059	0.0161	0.0194
All Platforms			
Mean	34,197	9983	4757
SD	1581	788	504
CV	0.0462	0.0789	0.1059

Note: The mean, standard deviation (SD), and coefficient of variation (CV) from triplicate Cytotrol™ cells stained with the same clone and lot of antibody (CD4 PE clone 13B8.2, CD19 PE clone J3-119, and CD22 clone SJ10.1H11) from Beckman Coulter using cocktails prepared in two different laboratories and acquired on four different flow cytometry platforms (2 FACSCANTO instruments in Laboratory 1, Attune NxT and CytoFlex LX cytometers in Laboratory 2).

Equation 1 are without F/P ratio adjustment. Again, the ABC values based on CD4 reference are very consistent for the unimolar CD20 PE and fairly comparable for the off-the-shelf CD20 PE across instruments (Table 7) but the values are decreased for the off-the shelf antibody in comparison to the unimolar preparation.

The two quantification approaches, QuantiBrite PE calibration and CD4 as a known reference marker enable quantitative measurement of CD20 expression levels in cytometer platform independent manner. In addition the value of correcting ABC values using the F/P ratios was examined, however, this correction requires the F/P ratios from the manufacturer and is based upon the assumption that the F/P ratios are accurate and explainable. These F/P ratios are obtained by absorbance measurements and are averaged results from bulk solutions. The same F/P ratio of two antibody solutions does not mean equivalence of the molecular constituents of the two solutions, for example, unlabeled, singlely labeled, and doublely labeled antibodies. Correction of ABC values using the F/P ratio did not improve the comparability and it can be concluded from the study that this additional calculation is not useful and hence not needed.

Since the three antibodies, CD4 PE, CD20 PE, and CD20 PE 1:1 are all produced by BD Biosciences, the PE conjugation processes are very similar except for narrower sized band collection for purifying CD20 PE 1:1 relative to CD20 PE and their F/P ratios provided by the vendor are shown to be similar. The Equation 1 above can be simplified without application of QuantiBrite PE calibration to both CD4 and CD20,

$$\mathrm{ABC}_{\mathrm{CD20}} = \frac{\mathrm{GeoMFI}_{\mathrm{CD20}}}{\mathrm{GeoMFI}_{\mathrm{CD4}}} \times 40{,}000. \quad (2)$$

The CD20 ABC values obtained by using Equation 2 are provided in the 4th column and 7th columns of Table 8 for the use of the off-

TABLE 6 Comparison of CD4 Raw GeoMFI and ABC value variability across instruments.

Flow cytometer	CD4 raw GeoMFI	CD4 ABC value
CytoFLEX LX		
Mean	75,294	24,472
SD	1204	385
CV	0.0160	0.0157
Attune NxT		
Mean	47,602	23,129
SD	363	181
CV	0.0076	0.0078
CANTO		
Mean	18,717	25,166
SD	29	39
CV	0.0015	0.0015
Lyric		
Mean	27,134	26,089
SD	509	488
CV	0.0188	0.0187

Note: The mean, standard deviation (SD), and coefficient of variation (CV) from triplicate Cytotrol™ cells stained with CD4PE, clone SK3 and CD45 v500, clone H130 (BDIS). No F/P ratio adjustment was made to the ABC values.

the-shelf CD20 PE and unimolar CD20 PE conjugate, respectively. By comparing the %CV associated with the mean ABC value across instruments, the simplified quantification approach using Equation 2 is as good as the approach using Equation 1. The use of CD4 as the reference marker does minimize the variability in the determination of the CD20 ABC values compared to the QuantiBrite PE calibration method.

4 | DISCUSSION

If ABC values are to be clinically relevant, they need to be independent of cytometers used and reproducible across flow cytometry instruments, laboratories, and time. Furthermore, minimizing variability in antigen quantification is crucial in large clinical studies of antigen directed therapies that examine the utility of expression levels of target antigens in predicting outcome. The same results must be generated by all participating laboratories. The ability to detect variability in results is especially important when monitoring changes in antigen expression overtime because decreased antigen expression serves as a potential indicator of loss of therapeutic efficacy. Methods of assessing interlaboratory and interinstrument variability are therefore needed. In addition, laboratories quantifying antigen expression need quality control reagents to evaluate new antibody lots, cocktail preparation, comparability of multiple intralaboratory flow cytometers, instrument performance over time, and determine the optimal antibody clone when setting up an assay. We studied the use of Beckman-Coulter Cytotrol™ control cells, a known stable and uniform lyophilized PBMC control, for quality control processes and evaluation of possible interlaboratory and cross platform variability. We found that a stable control cell product such as Cytotrol™ is highly useful in validating synchronization of multiple intralaboratory instruments to produce consistent results (Table 1). In addition, shelf stable control cells provided an excellent identical specimen for evaluation of instrument function over time (Table 2). Shelf stable control cells provide an excellent quality control product for evaluating new antibody lots, which may be superior since it does not rely on comparison to an old antibody lot that may have degraded over time (Table 3). Lastly, Cytotrol™ cells served an excellent Quality Control reagent for double checking cocktail proparation by different individuals.

When designing a flow cytometry assay the choice of antibody clone is important. Our studies indicate it is especially critical in assays that measure levels of antigen expression. While the two CD4 clones were similar, notable differences were observed between the CD19, CD20, and CD22 clones (Table 4). It should be noted that the observed differences in these antibody clones may be secondary to different antigen epitopes being targeted by the clones and possible differential preservation of epitope sites during lyophilization. Confirmation in biological samples without lyophilization is therefore recommended. When validating an assay run in multiple institutions, cocktail preparation and flow cytometer platform are possible sources of variability in the data collected. Mimicing these conditions, Cytotrol™ cells were stained with cocktails prepared separately by the two participating laboratories using the same lots of reagents, CD4 PE, CD19 PE, and CD22 PE and run on four different flow cytometer platforms. Consistent ABC values were obtained across instrument platforms, as expected (Table 5). Use of an identical control cell product allowed confident assessment of potential variability between the two laboratories and different platforms.

From the CD4 GeoMFI values measured using the same sample preparation on different cytometers shown in Table 6, it is clear that instrument-dependent GeoMFI cannot be used to report the expression levels across instruments and laboratories. To quantify antigen expression levels in a cytometer independent manner, a well-adapted antigen quantification scheme utilized in clinical laboratories relies on the use of Quantibrite PE beads and PE labeled antibodies (Davis et al., 1998; Salem et al., 2018; Tembhare et al., 2013). The method enables antigen expression analysis in the PE channel with an assumption that the absorption and fluorescence properties of QuantiBrite PE beads and PBMC stained with PE labeled antibodies are the same. Because cytometer platforms used in this study have different optical configurations, including laser wavelength and bandpass filters, PE fluorescence spectra of QauntiBrite PE beads and PE labeled PBMC were measured to verify this assumption. The closely matched PE fluorescence spectra of the two samples with different bandpass filters and under either 488 or 561 nm laser excitation (Figure 1) support that the ABC values based on the QuantiBrite PE calibration method should be comparable across different cytometer platforms used.

Although the QuantiBrite PE calibration assay can be easily utilized in a clinical laboratory for reproducible results allowing

TABLE 7 Comparison of CD20 ABC variability using the off-the-shelf (OTS) and unimolar (U) CD20 PE conjugates across instruments.

Conjugate/Mfr.	Raw GeoMFI	ABC value[a]	ABC value with F/P corrected	$(ABC_{CD20}/ABC_{CD4}) \times 40{,}000$ (CD4 ABC from Table 8 as a reference marker)[a]
CytoFLEX LX				
CD20 OTS, [F/P = 0.946]: Mean (SD) [CV]	34,123	11,247	11,889	18,384
	(1014)	(328)	(347)	(1074)
	[0.0297]	[0.292]	[0.0292]	[0.0584]
CD20 U, [F/P = 1.094]: Mean (SD) [CV]	50,782	16,843	17,550	27,530
	(692)	(222)	(235)	(363)
	[0.0136]	[0.0132]	[0.0134]	[0.0132]
Attune NxT				
CD20 OTS: Mean (SD) [CV]	17,028	8063	8524	13,945
	(3649)	(1767)	(1869)	(3058)
	[0.2143]	[0.2191]	[0.2193]	[0.2193]
CD20 U, [F/P = 1.094): Mean (SD) [CV]	31,961	15,373	16,234	26,587
	(2129)	(1049)	(1108)	(1815)
	[0.0666]	[0.682]	[0.0682]	[0.0683]
Canto II				
CD20 OTS, [F/P = 0.946]: Mean (SD) [CV]	6158	8358	8835	13,285
	(1711)	(2301)	(2433)	(3658)
	[0.2778]	[0.2753]	[2754]	[0.2753]
CD20 U, [F/P = 1.094): Mean (SD) [CV]	12,402	16,735	17,672	26,600
	(803)	(1075)	(1135)	(1709)
	[0.0647]	[0.0642]	[0.0642]	[0.0642]
Lyric				
CD20 OTS, [F/P = 0.946]: Mean (SD) [CV]	11,916	11,469	12,124	17,584
	(224)	(216)	(224)	(343)
	[0.0188]	[0.0188]	[0.0185]	[0.0195]
CD20 U, [F/P = 1.094): Mean (SD) [CV]	17,964	17,282	18,249	26,497
	(1178)	(1132)	(1196)	(1799)
	[0.0656]	[0.0655]	[0.0655]	[0.0679]

[a]Indicates no F/P ratio adjustment. The mean, standard deviation (SD), and coefficient of variation [CV] from triplicate Cytotrol™ cells stained with off-the-shelf CD20 PE, clone L27 and CD45 v500, clone H130 or a second cocktail with unimolar CD20 PE, clone L27 and CD45 v500, clone H130, both from BD Bioscences.

TABLE 8 Comparison of CD20 ABC variability using three different quantification approaches across instruments.

	CD20 OTS ABC	$(ABC_{CD20OTS}/ABC_{CD4OTS}) \times 40{,}000$	$(GeoMFI_{CD20OTS}/GeoMFI_{CD4OTS}) \times 40{,}000$	CD20 1:1 ABC	$(ABC_{CD201:1}/ABC_{CD4OTS}) \times 40{,}000$	$(GeoMFI_{CD201:1}/GeoMFI_{CD4OTS}) \times 40{,}000$
CytoFlex LX	11,247	18,384	18,128	16,843	27,530	26,978
Attune NxT	8063	13,945	14,308	15,373	26,587	26,856
CANTO	8358	13,285	13,160	16,735	26,600	26,505
Lyric	11,469	17,584	17,872	17,282	27,469	26,482
Mean	9784	15,800	15,867	16,558	26,956	26,705
SD	1824	2558	2509	825	436	250
CV	0.186	0.162	0.158	0.050	0.016	0.009

Note: CD20 OTS: Non-unimolar off the shelf CD20. CD20 1:1: Unimolar CD20. ABC values are from Table 7 and are not corrected by the F/P ratio.

comparison of antigen expression among patients and detection of changes in levels of antigen expression in response to therapy, it is not without limitations. The method only allows expression analysis in the PE channel and has issues associated with lot-to-lot variability on antibody PE conjugates (Table 3) (Wang et al., 2011) as well as limited availability of high quality unimolar antibody-PE conjugates such as CD20, CD38 and HLA-DR. It is evidentally clear that the use of off-the-shelf antibody PE conjugates (CD20 OTS) will result in lower ABC values of antigens than the use of unimolar antibody conjugated with PE (CD20 U) as shown in this study. An alternative antigen quantification scheme reported in the literature (Degheidy et al., 2016; Wang et al., 2016) is to use a biological cell reference, which is known to possess a fixed number of known biomarkers such as CD4 on Cytotrol™ control cells characterized by using three orthogonal methods, quantitative flow cytometry, mass cytometry, and mass spectrometry. This approach enables antigen expression analysis in different fluorescence channels (Degheidy et al., 2016). Because the quantification method involves both antibodies for reference marker and antigen of interest, it is recommended to utilize two antibodies produced by the same manufacturer to ensure a consistent antibody-fluorophore conjugation process for the two labeled antibodies. The source of variances on the CD4 based quantification scheme was investigated and reported in the previous study that showed relatively small uncertainty in CD19 quantification using CD4 as a reference marker (Wang et al., 2021). Because no uncertainty was provided for the number of PE molecules associated with each Quantibrite PE bead population, the variance with the use of Quantibrite PE calibration could not be assessed in that study.

Our results on the CD20 expression analysis on Cytotrol™ cells using Equation 1 shown in Table 7 and Equation 2 in Table 8 demonstrate that the antigen quantification method based on CD4 as the reference marker performs well with no larger uncertainty compared to the Quantibrite PE calibration approach. The accurate level of CD20 expression, however, is likely higher than the values obtained on the basis of QuantiBrite PE calibration. An antibody with a F/P ratio of close to 1 still contains antibodies without fluorophore label, labeled with a single PE, or labeled with ≥2 PE molecules, and that unlabeled antibody has a much higher binding affinity to antigen. This likely contributes to the variable results obtained with different antibody lots. Having a stable control to compare antibody lots is vital for longitudinal studies of antigen expression. Importantly, this study focuses on the comparability and reproducibility of antigen expression analysis across different cytometer platforms and laboratories rather than the accuracy/trueness of the biomarker expression levels. If the goal of an assay is to accurately determine the number of antigen molecules residing on the cell surface an unimolar antibody should be used. As antigen directed therapy expands it is hoped that manufacturers provide more high quality unimolar antibody preparations for antigen quantification but until such products become available, differences in antigen expression between patients or over time in the same patient can be determined using ABC values and off-the-shelf antibodies with an F/P ratio close to one with minimal variability demonstrated by accurate lot testing. It is worth mentioning that CD20 quantification was demonstrated previously using CD4 on Cytotrol™ as the reference marker in a single-tube assay format where both stained specimen and Cytotrol™ were added in the same tube (Wang et al., 2016). Spiking a single PE calibration bead, either a part of QuantiBrite PE bead kit or a different PE calibration bead material such as PE calibration beads made by Slingshot Biosciences and BD's FC beads PE, into the specimen could help to evaluate the precision of antigen expression analysis.

In conclusion, as response to antigen based immunotherapy can rely upon the level of antigen expression by tumor cells and as decreasing levels of antigen expression can be an early indicator of developing resistance to therapy, a reliable antigen quantification assay is needed. Due to the ease of use and reliability of the QuantiBrite assay as well as its general utility in quality control, determination of ABC values can meet this need. As in all flow cytometric assays, the quality of data is dependent upon the quality of the antibodies used and unimolar antibodies, if available, are preferable. On the other hand, if antigen quantification is needed in non-PE channel, CD4 reference marker approach with the use of Equation 2 serves the quantification purpose. If this assay is to be utilized in a clinical setting quality control methods have to be instituted to assure reproducibility and allow validation across laboratories. We have demonstrated in this study that use of a lyophilized cell control is highly valuable in achieving these goals.

ACKNOWLEDGMENTS

Research reported in this publication was supported in part by the intramural program, COCR, the National Cancer Institute, the National Institutes of Health and the Biosystems and Biomaterials Division, National Institute of Standards and Technology.

DISCLAIMER

To specify an experimental procedure as completely as possible, certain commercial materials, instruments, and equipment are identified in this manuscript. In no case does identification of the manufacturer of particular equipment or materials imply a recommendation or endorsement by the National Institute of Standards and Technology or the National Institutes of Health, nor does it imply that the materials, instruments, and equipment identified are necessarily the best available for the purpose.

ORCID

Constance Yuan https://orcid.org/0000-0002-2601-3665
Hao-Wei Wang https://orcid.org/0000-0002-5408-9253

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How to cite this article: Tian, L., Nelson, A. R., Lowe, T., Weaver, L., Yuan, C., Wang, H.-W., DeRose, P., Stetler-Stevenson, M., & Wang, L. (2024). Standardization of flow cytometric detection of antigen expression. *Cytometry Part B: Clinical Cytometry*, 1–10. https://doi.org/10.1002/cyto.b.22155