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Original Article

Consistent, Multi-Instrument Single Tube Quantification of CD20 in Antibody Bound per Cell Based on CD4 Reference

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Detecting changes in the expression levels of cell antigens could provide critical information for the diagnosis of many diseases, for example, leukemia, lymphoma, and immunodeficiency diseases, detecting minimal residual disease, monitoring immunotherapies and discovery of meaningful clinical disease markers. One of the most significant challenges in flow cytometry is how to best ensure measurement quality and generate consistent and reproducible inter-laboratory and intra-laboratory results across multiple cytometer platforms and locations longitudinally over time. In a previous study, we developed a procedure for instrument standardization across four different flow cytometer platforms from the same manufacturer. CD19 quantification was performed on three of the standardized instruments relative to CD4 expression on T lymphocytes with a known amount of antibody bound per cell (ABC) as a quantification standard. Consistent and reliable measures of CD19 expression were obtained independent of fluorochrome used demonstrating the utility of this approach. In the present investigation, quantification of CD20 relative to CD4 reference marker was implemented within a single tube containing both antibodies. Relative quantification of CD20 was performed using anti-CD20 antibody (clone L27) in three different fluorochromes relative to anti-CD4 antibody (clone SK3). Our results demonstrated that cell surface marker quantification can be performed robustly using the single tube assay format with novel gating strategies. The ABC values obtained for CD20 expression levels using PE, APC, or PerCP Cy5.5 are consistent over the five different instrument platforms for any given apparently healthy donor independent of the fluorochrome used. © 2015 International Clinical Cytometry Society

Key terms: flow cytometry; CD20 quantification; instrument characterization and standardization; mean/geometric mean fluorescence intensity; antibody bound per cell

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Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

Flow cytometry is a powerful immunological technique and is able to assess complex biological processes at a single cell level in heterogeneous populations. Cells can be obtained from different sources including blood, lymph nodes, bone marrow, and other biological sources. Multiparameter flow cytometer assays are routinely used in clinical laboratories to measure the cell number of specific immunophenotypes and to estimate expression levels of specific receptors/antigens either on the cell surface or intracellularly. The cell number and specific receptors/antigens serve as biomarkers for pathological conditions at various stages of a disease. Flow cytometry measurements are also used to support many clinical, pharmacologic, calibration and measurement capability claims used for drug, device, and biologics product development, approval, and clearance.

Quantitative flow cytometry plays an increasingly important role for the diagnosis of hematologic malignancies and immune disorders (1–5). An ultimate goal of quantitative flow cytometry is to measure the number of antigens or ligand binding sites associated with a cell through the measurement of the number of antibody bound per cell (ABC; (5–8)). It is well known that the use of QuantiBrite™ phycoerythrin (PE) calibration beads combined with unimolar monoclonal antibody PE conjugates enables the quantitation of antigen expressions (9–11). However, issues such as the lot to lot quality of unimolar PE antibody conjugates (11) and the availability of unimolar conjugates for biomarkers other than CD4, CD20, CD38, and HLA-DR are not yet resolved. Additionally, this calibration scheme only deals with the quantitation associated with the PE fluorescence channel. Another way to accomplish the goal of cell surface marker quantification is to use a biological reference cell, which is known to possess a fixed number of well characterized protein biomarkers such as CD4 (12–17). An international cell reference material of lyophilized peripheral blood mononuclear cells (PBMC) prelabeled with anti-CD4 fluorescein isothiocyanate (FITC) antibody has recently come out for calibration and standardization of CD4+ cell enumeration for quality assurance of HIV/AIDS monitoring as well as quantitative expression analysis in the FITC channel (16,17). This cell reference material only enables quantitative expression analysis in the unit of equivalent fluorescein fluorophore (EFF) because the link between EFF and ABC has not yet been established. Moreover, guidelines for qualifying biomarker quantification using cell-based fluorescence assays (18,19) are emerging. A common, stabilized reference material with centralized value assignments is critically needed for quantitative flow cytometry, which requires strong and cohesive support of all authoritative standards bodies including National Institute of Standards and Technology (NIST), International Society for the Advancement of Cytometry, International Council for Standardization in Haematology, Clinical and Laboratory Standards Institute, and International Clinical Cytometry Society. After linearity check of the fluorescence channels, a comparison of the fluorescence intensities from the reference biomarker and the test biomarkers can provide an estimate of ABC for the test biomarkers in different fluorescence channels (15). Importantly, this approach enables biomarker quantification in all fluorescence channels of flow cytometers.

Multicenter biomarker quantitative studies have not yet been reported widely, likely due to the difficulty introduced by using various flow cytometer platforms. However, commercial kits for red cell CD55 and CD59 (Biocytex) and neutrophil CD64 [Leuko64 (CD64 and CD163) from Trillium Diagnostics] are examples where quantitative molecular expression measurements have been achieved on the basis of internal bead standards for assay value assignments. Furthermore, a few monocyte HLA-DR multicenter studies have been successfully conducted using QuantiBrite HLA-DR/Monocyte reagent and QuantiBrite PE calibration beads (1,20). Very recently, we have successfully demonstrated the feasibility of this type of study using four different flow cytometer platforms from the same vendor with somewhat different optical configurations at two different locations (15). This type of study requires both multiplatform instrument standardization and the use of a biological reference biomarker with a known expression level for biomarker quantification. In that study, we optimized a procedure for instrument standardization across different flow cytometer platforms and then carried out CD19 quantification relative to CD4 reference biomarker. The comparable results of CD19 expression in the unit of ABC from two apparently healthy donors (AHD) on three different instruments and two different fluorophore labels support the biomarker quantification approach that relies on instrument standardization and the use of CD4 reference biomarker. The use of a CD4 reference biomarker enables the measurement of CD19 expression in ABC units with significantly lower coefficient of variation (CV) across different cytometer platforms, when compared with the CV of the geometric mean fluorescence intensity (MFI) for CD19 expression after across platform instrument standardization.

In the same study, we operated under the assumption, supported by our previous study results (21), that different antibodies against different antigens, for example, reference biomarker CD4 and test biomarker CD19, with the same fluorochrome label likely have very similar average equivalent fluorescence per antibody values as long as they are produced by the same manufacturer under rigorous current good manufacturing practice (cGMP).

CD20 is a well-known marker for B lineage cells and has been useful in studies of B-cell chronic lymphocytic leukemia (22–25). In this study, we extended our method of relative quantification in two significant ways. First, we used both a single tube assay (staining of CD4 and CD20 simultaneously in the same tube, which is preferable for processing clinical samples) as compared with a two-separate tube assay [staining of CD4

and CD20 in two separate tubes; using the same staining protocol as used in the previous study of CD19 quantification (15)]. The use of the single tube assay avoids sources of error causing result variability, for example, the amount of sample and staining reagents added and possible instrumental variation. These methods gave equivalent results and showed that the two different antibodies labeled with the same fluorochrome can have similar equivalent fluorescence per antibody and be used to provide quantification of the test biomarker (CD20 on B cells) relative to a reference marker (CD4 on T cells) in a single blood sample. Second, we showed that the test biomarker can give consistent quantitative results using three different fluorochromes of anti-CD20 antibody (clone L27) relative to an anti-CD4 antibody (clone SK3).

MATERIAL AND METHOD

Reagents

Heparinized anonymous AHDs' samples were obtained from NIH's Department of Transfusion Medicine according to their institutional review boards guidelines. Monoclonal antibodies, CD4 PE (clone SK3, Catalog Number: 347327), CD4 peridinin–chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5; clone SK3, Catalog Number: 341654), CD4 APC (clone SK3, Catalog Number: 340443), CD4 allophycocyanin-cyanine 7 (APC-Cy7; clone SK3, Catalog Number: 341095), CD4 AmCyan (clone SK3, Catalog Number: 339187), CD4 phycoerythrin-cyanine 7 (PE-Cy7; clone SK3, Catalog Number: 348789), CD4 V450 (clone SK3, Catalog Number: 651850), CD20 PE (clone L27, Catalog Number: 346595), CD20 APC (clone L27, Catalog Number: 340941), CD20 PerCP-Cy5.5 (clone L27, Catalog Number: 340955), CD45 AmCyan, CD3 APC-Cy7, and CD19 V450 were purchased from BD Biosciences (San Jose, CA).

Sample Staining Procedure

Whole blood samples were washed twice with phosphate-buffered saline (PBS), pH.7.4 containing 2% FBS (Sigma-Aldrich, St Louis, MO) to remove cytophilic antibody (26). A 200 μL of whole blood samples was stained with a cocktail of the following antibodies under saturation conditions: CD45 AmCyan, CD3 APC-Cy7, CD19 V450, and CD4/CD20 (either labeled with PE, APC, or PerCP-Cy5.5) for 30 min at room temperature. Staining of CD20 and reference marker CD4 was carried out either in the same tube or in two separate tubes. The red blood cells were subsequently lysed with 1× FACS Lysing Solution (BD Biosciences) according to the manufacturer's recommendations. After centrifugation, the cell pellet was washed twice with PBS/2% FBS (pH7.4) and resuspended in a 0.5 mL of PBS with 1% paraformaldehyde pH 7.4. Separately, unstained cells as well as single stained tubes for CD4 FITC, CD4 PE, CD4 APC, CD4 PerCP-Cy5.5, CD4 APC-Cy7, CD4 AmCyan, CD4 V450, were used to setup flow cytometric compensation.

Multiplatform Instrument Standardization

Five flow cytometry instruments from the same manufacturers in two different laboratories were used in this study. Instrument 1 (FACSCanto II), Instrument 2 (Fortessa), Instrument 3 (LSRII), Instrument 4 (FACSAria III), and Instrument 5 (FACSAria II) are all from BD Biosciences (San Jose, CA). The first four instruments are located at Food and Drug Administration and the fifth instrument is located at NIST. The configurations of the five instruments including the lasers and optical filters are provided in the Supporting Information Tables S1 and S2. Cytometer Setup & Tracking (CS&T) beads (Lot #: 81638), composed of equal concentrations of dim, mid, and bright dye embedded polystryrene beads [2 μm (dim bead), 3 μm (mid and bright beads)], were from BD Biosciences and used throughout the study to define the base line and to perform daily QC/QA for each instrument. Similar to the criteria illustrated in the exploratory study (15), a target cytometer was determined across the five instrument platforms on the basis of the largest value of the electronic noise robust standard deviation (rSD_{EN}, for definition, see the whitepaper at www.bdbiosciences.com/documents/Robust_Statistics_in_BDFACSDiva.pdf) and lowest linearity maximum (Lin Max) for all eight detectors. Since CD20 quantification would be carried out on three different fluorescence channels, PE, PerCP Cy5.5 and APC, the rSD_{EN} and Lin Max of these three channels were the determining factors for the choice of the target instrument (Supporting Information Table S3). For this study, Instrument 1 was identified as the least sensitive cytometer or the target cytometer.

An unstained donor sample obtained from an apparently healthy individual was used to adjust PMT voltage for each fluorescence detector of Instrument1 such that the rSD_{EN} of autofluorescence signal from unstained cells is within 2.5–3 times the rSD_{EN} of that detector shown in the cytometer baseline report. The optimized PMT voltages were further verified for each detector using both singly stained CD4 blood control and multicolor stained tube to ensure that the brightest populations were not out of the dynamic range of each detector. Moreover, the MFI values of the mid CS&T bead population were determined and served as the target MFI values that were transferred to the other four flow cytometers. The PMT voltages on the other four flow cytometers were adjusted in such a way that the CS&T mid peaks have nearly equal target MFIs on the standardized target instrument. These optimized PMT voltages were further checked using the single stained tubes and multicolor test tubes and used for subsequent acquisition. After the cytometers were standardized, Ultra Rainbow beads (Catalog #: URCP-38-2K), containing a blank and five different fluorescence intensity populations from Spherotech Inc (Lake Forest, IL), were run to further ensure the linearity of the fluorescence channels used for biomarker quantification in the unit of equivalent reference fluorophore (ERF; (14)).

After adjusting the voltage of each channel for each instrument based on the target MFI value, we then run the same compensation controls independently on each instrument to obtain a compensation matrix for each flow cytometer. The compensation matrix is different from each instrument due to different instrument design and optical configuration. New compensation controls were prepared for each experiment performed on different dates. Performing compensation on different dates under the identical instrument condition (same lasers, optical assignment, PMTs, and filter sets) allows monitoring the consistency of compensation matrixes obtained at different times.

CD20 Quantification Scheme

Once the linearity of the fluorescence channel used for quantifying CD20 expression level is assured with the use of Ultra Rainbow beads in the unit of EFF, the relative quantification approach is exemplified in Figure 1 using CD20 PE as an example. With CD4 PE serving as a reference biomarker with a known ABC value of ~40,000 for fixed whole blood using the off-the shelf CD4 PE (SK3 clone) reagent (10,13,21,27), the linear EFF calibration scale (left Y-axis in Fig. 1) is transformed to the ABC scale (right Y-axis). A relative ABC value for CD20 expression by means of CD20 PE is then calculated practically as

$$\text{relABC}_{\text{CD20}} = \frac{\text{MFI}_{\text{CD20}}}{\text{MFI}_{\text{CD4}}} \times \text{ABC}_{\text{CD4}} \qquad (1)$$

with an assumption that the effective fluorescence per antibody for anti-CD4 and anti-CD20 made by the same manufacturer is the same (15,21). The ABC value of ~40,000 for fixed CD4+ lymphocytes from AHD whole blood is consistent with the observations of very similar MFI values for fixed healthy whole blood samples and for Cyto-Trol control cells using the off-the-shelf anti-CD4 antibodies (SK3 clone), and the CD4 receptor protein density divided by two for Cyto-Trol cells because each anti-CD4 antibody (SK3 clone) binds two CD4 receptor proteins (13). The CD4 receptor protein density on CD4+ lymphocytes from Cyto-Trol cells has been measured by using quantitative mass spectrometry (27). On the basis of the EFF calibration, the ratio of MFI_{CD20} and MFI_{CD4} is similar to the ratio of EFF_{CD20} and EFF_{CD4} when the intercept of the calibration line is small.

RESULTS AND DISCUSSION

Gating Strategy

CD20 quantification relative to CD4 reference marker was carried out in PE, APC and PerCP Cy5.5 channels using two different assay formats, a single tube assay, and a two separate tube assay across five different cytometer platforms. The gating strategies for measuring CD20 and CD4 expression levels as illustrated in Figure 2 using a single tube assay containing both markers stained in PE are summarized as follow: Firstly, a general

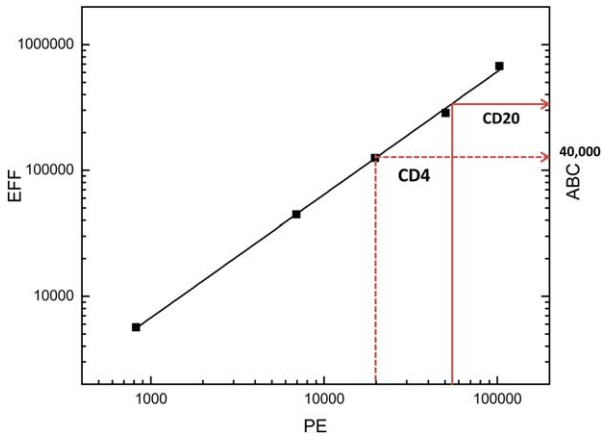

FIG. 1. Schematic of CD20 quantification on PE channel. The linearity of PE channel is assured by using Ultra Rainbow beads for the calibration in the unit of the EFF (14; square symbol). The adjusted R-squared for the linear fit to the log transformed data as shown is 0.998. Using CD4 PE (clone SK3) as a reference biomarker with a known ABC value of 40,000 for fixed whole blood, the CD20 expression level in ABC unit is determined on the basis of Eq. (1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

lymphocyte gate was drawn as R1 in CD45 vs. SSC-A (SSC-Area) (A). Under R1, a refined lymphocyte gate was drawn as R2 in the dot plot of FSC-A (FSC-Area) vs. SSC-A (B). While gated on R1 + R2, T-cell (R3), and B-cell (R4) gates were created using a plot of CD3 vs. CD19 (C). Under R1 + R2 + R3 (T-cells), plots of FSC-A vs. FSC-H (FSC-Height) (D) and SSC-A vs. SSC-H (SSC-Height) (E) were used to identify the singlet gates for T cells. A histogram of CD20/CD4 PE gated on T cells shows CD4+ T cells and CD4− T cells (F). Separately, under R1 + R2 + R4 (B-cells), plots of FSC-A vs. FSC-H (G), and SSC-A vs. SSC-H (H) were used to identify the singlet gates for B cells. The histogram of CD20/CD4 PE gated on B cells shows CD20+ B cells (I).

A different gating strategy can also be used for the analysis of CD4 and CD20 expressions in the single tube assay. While gated on R2, the histogram of CD20/CD4 PE displays both CD4+ and CD20+ cells in a gate R5 (J). Under the gate R5, CD4+ T cells (CD3+), and CD20+ B cells (CD3−) were identified using the histogram of CD3 APC Cy7 (K). Finally, the single color histogram for CD20 and CD4 expressions in PE (L) shows the overlay of CD20+ B cells (in red, from B cell gate in K), CD4+ T cells (in blue, from CD4+ve gate in K), and CD4− T cells (in green). The histogram L can also be derived from F/I of the first gating strategy described above. Geometric mean fluorescence intensities (MFIs) identified for CD20+ cells and CD4+ T cells were then used for subsequent ABC value determination.

Validation of a Single Tube Quantification Assay by a Two Separate Tube Assay

Geometric MFI values for CD20 PE (clone L27), CD4 PE (clone SK3), and ratio of their respective MFI values are provided in Table 1. The MFIs of both CD20 PE and

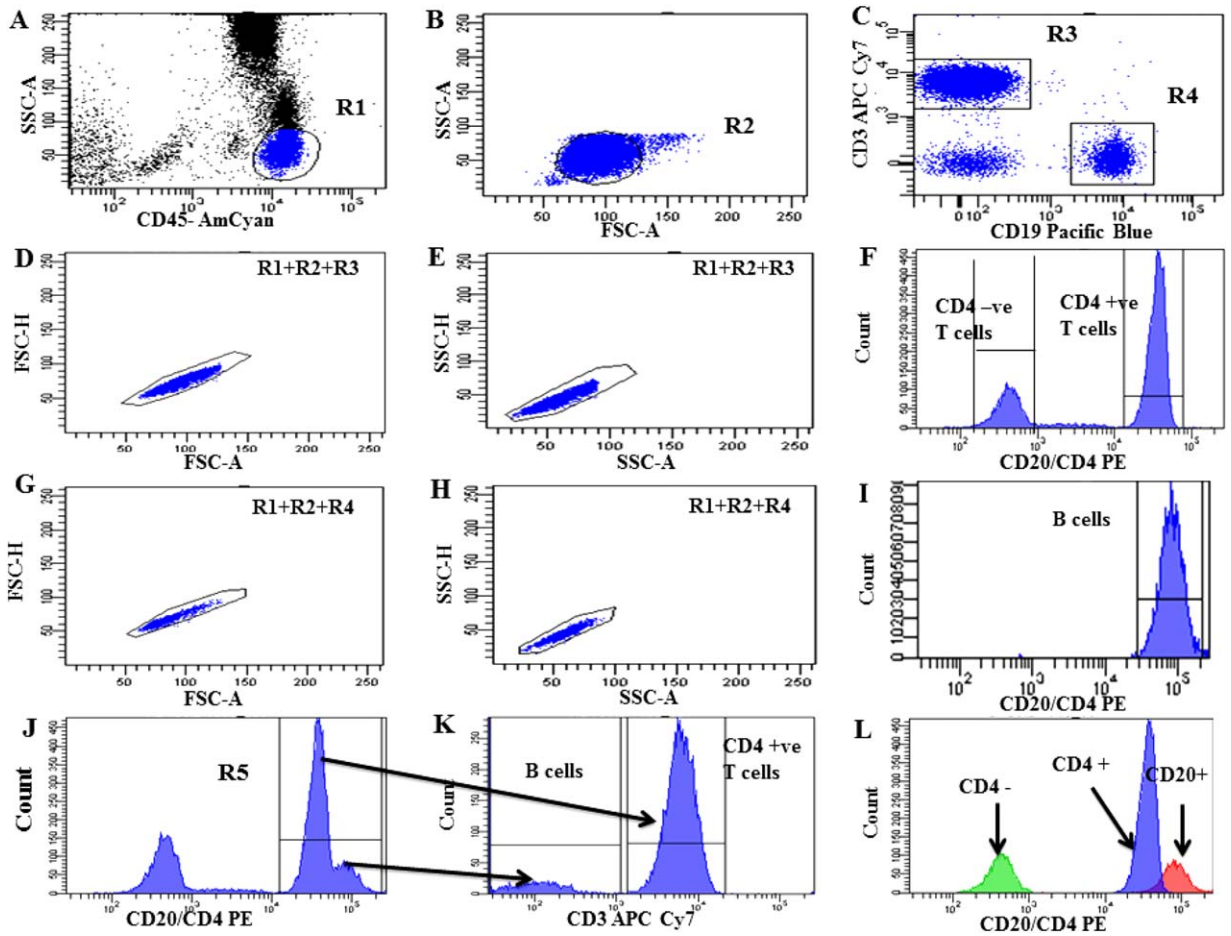

FIG. 2. Gating strategy for measuring CD20 (on B cells) and CD4 (on T cells) expression levels in the single tube of blood containing both markers stained in PE: (**A**) CD45 vs. SSC plot to select a generous lymphocyte region (R1) gate; (**B**) gated on R1, a refined lymphocyte gate (R2) was generated using a plot of FSC-A vs. SSC-A; (**C**) T-cell (R3) and B-cell (R4) gates were created using a plot of CD3 vs. CD19 gated on R1 + R2; (**D and E**) gated on R1 + R2 + R3 (T-cells), plots of FSC-A vs. FSC-H in **D** and SSC-A vs. SSC-H in **E** were used to identify the singlet gates for T cells; (**F**) histogram of CD20/CD4 PE gated on T-cells shows CD4+ T cells and CD4− T cells [Geometric Mean Fluorescence Intensities (MFIs) were obtained for CD4+ T cells for subsequent ABC value determination]; (**G and H**) gated on R1 + R2 + R4 (B-cells), plots of FSC-A vs. FSC-H in **G** and SSC-A vs. SSC-H in **H** were used to identify the singlet gates for B cells; (**I**) histogram of CD20/CD4 PE gated on B-cells allows MFIs to be obtained for CD20+ B cells for subsequent ABC value determination. A different way is shown in **J, K, and L** for the analysis of CD4 and CD20 expression in the same tube: (**J**) histogram of CD20/CD4 PE gated on R2 in (**B**) displays both CD4+ and CD20+ cells in a gate R5; (**K**) Gated on R5, CD4+ T cells (CD3+ve), and B cells (CD3–ve) were identified using the histogram of CD3 APC Cy7; (**L**) a single color histogram for CD20 and CD4 expressions in PE shows the overlay of CD20+ B cells (in red), CD4+ T cells (in blue) and CD4− T cells (in green). The MFIs were then identified for CD20+ cells and CD4+ T cells for subsequent ABC value determination. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CD4 PE show large variations from one instrument to another for any given single donor even though all five flow cytometers were standardized. For instance, the MFI of CD20 PE for AHD-3 from Cytometer #4 is more than twice of the MFI from Cytometer #1. However, the MFI ratios of CD20/CD4 across instrument platforms in both assay formats are consistent. Since the CD20/CD4 ratios for AHD-2 show the largest variations among the five AHDs, a paired *t* test was performed on these two sets of ratio values from the two assay formats; the difference between the two sets of values is considered to be not statistically significant at 95% confidence level. The ratio of CD20 and CD4 multiplied by a constant, the ABC value of CD4, as shown in Eq. (1) gives the relative ABC value of CD20 expression. The mean CD20/CD4 ratio and its CV% are also given in the Table 1 for each AHD across five instrument platforms showing statistically identical between the single tube assay and two separate tube assay formats. Unlike our previous study in which CD19 quantification was performed only in two separate tube assay format (15), this study demonstrates biomarker quantification can be performed simultaneously and robustly using the single tube assay.

Quantification of CD20 Expression Level in ABC Using Anti-CD20 Antibody (Clone L27) with Three Different Fluorochrome Labels

Applying the single tube assay format and gating strategies shown in Figure 2, quantification of CD20 expression level in ABC was carried out using three different

Table 1
Geometric MFI Values for CD20 PE (clone L27), CD4 PE (clone SK3), and Ratio of MFI Values of CD20 and CD4 using Two Different Assay Formats, a Single Tube Assay, and a Two-Separate Tube Assay, on 5 AHD, and Five Different Cytometer Platforms

		AHD-1	AHD-2	AHD-3	AHD-4	AHD-5
Single tube assay						
Cytom 1	CD20	62,865	63,051	63,612	81,124	78,105
	CD4	28,582	28,171	28,081	35,816	30,883
	CD20/CD4	2.20	2.24	2.27	2.26	2.53
Cytom 2	CD20	74,200	87,822	84,576	68,257	75,974
	CD4	34,567	35,340	36,006	30,911	27,268
	CD20/CD4	2.15	2.48	2.35	2.21	2.79
Cytom 3	CD20	59,269	85,899	83,866	77,247	78,627
	CD4	27,091	35,191	34,016	33,008	28,381
	CD20/CD4	2.19	2.44	2.46	2.34	2.77
Cytom 4	CD20	104,897	121,423	132,915	126,840	130,690
	CD4	50,822	51,225	55,818	55,162	50,043
	CD20/CD4	2.06	2.37	2.38	2.30	2.61
Cytom 5	CD20	61,667	84,669	69,995	52,364	54,803
	CD4	29843	33,718	30,221	22,843	20,087
	CD20/CD4	2.07	2.51	2.32	2.29	2.73
Mean ratio (CV%)		2.13 (3.1%)	2.41 (4.5%)	2.36 (3.0%)	2.28 (2.1%)	2.69 (4.2%)
Two Separate Tube Assay						
Cytom 1	CD20	66,649	65,094	63,440	80,008	81,938
	CD4	29,498	28,853	29,001	35,167	31,039
	CD20/CD4	2.26	2.26	2.19	2.28	2.64
Cytom 2	CD20	76,550	89765	82,367	66,562	77,568
	CD4	35,890	36,544	34,461	29,842	29,764
	CD20/CD4	2.13	2.45	2.39	2.23	2.61
Cytom 3	CD20	55,290	85,215	84,944	76,048	79,903
	CD4	26,767	35,977	34,911	32,914	29,340
	CD20/CD4	2.07	2.37	2.43	2.31	2.72
Cytom 4	CD20	110,093	124,540	131,734	122,399	136,854
	CD4	53,047	52,621	59,969	54,094	52,056
	CD20/CD4	2.08	2.37	2.20	2.26	2.63
Cytom 5	CD20	60,160	87,249	67,912	51,545	55,542
	CD4	27,950	32,481	31,411	21,690	19,814
	CD20/CD4	2.15	2.68	2.16	2.38	2.80
Mean ratio (CV%)		2.14 (3.6%)	2.43 (6.5%)	2.27 (5.5%)	2.29 (2.5%)	2.68 (2.9%)

Mean CD20/CD4 ratio and its CV% are also calculated for each AHD across 5 instrument platforms in two assay formats. The CV of the ratio value per instrument platform from technical triplicates is no more than 1.5% for multiple samples.

fluorophore labels, PE, APC, and PerCP Cy5.5. As shown in Figure 3, dot plots of CD20/CD4 vs. CD3 display very similar patterns in all three fluorochromes over the five different flow cytometer platforms for a given AHD. This consistency allows for quantifying CD20 expression relative to CD4 reference marker in all three different fluorescence channels. Three distinct cell populations, CD20+ B cells (red dots), CD4+ T cells (blue dots), and CD4− T cells (green dots) are well separated for ABC value determination according to Eq. (1).

The ABC values obtained for CD20 expression on 10 AHDs are summarized in Table 2. For any given AHD, the ABC values are consistent over the five different instrument platforms and fluorochrome label used, even though large variations on MFI of CD20 were observed (Table 1). A paired *t* test on the two complete sets of ABC values of CD20 PE and CD20 PerCP Cy5.5 was performed; the difference between the two sets of values is considered to be not statistically significant at 95% confidence level. As shown in our previous study (15,21), these results from three different fluorochrome labels (Table 2) again provide evidence that supports the assumption that different antibodies against different antigens with the same fluorophore label can have similar average fluorescence per antibody values if antibodies are produced by the same vendor under rigorous cGMP. The consistency of the ratio values between the two assay formats has been observed using APC and PerCP Cy5.5 as labeling fluorophores (Supporting Information Table S4).

The CVs of interfluorophore ABC values are, in bulk, similar to the CVs of the interinstrument values in Table 2 (e.g., 0.4% CV of the interfluorophore value for AHD-7 on Cytom 5, 1.3% CV of the interfluorophore value for AHD-3 on Cytom 3, 1.1% CV for AHD-2 on Cytom 4). However, we observed a 9.0% CV for AHD-5 on Cytom 2. Since there are large variations seen with different antibody clones bearing the same fluorophore or different fluorophores because of the differences in fluorophore conjugation chemistries and purification methods from different vendors, we carefully designed this study using single anti-CD20 clone (L27) and single anti-CD4 clone (SK3), produced by the same manufacturer to minimize the variations due to antibody reagents. The

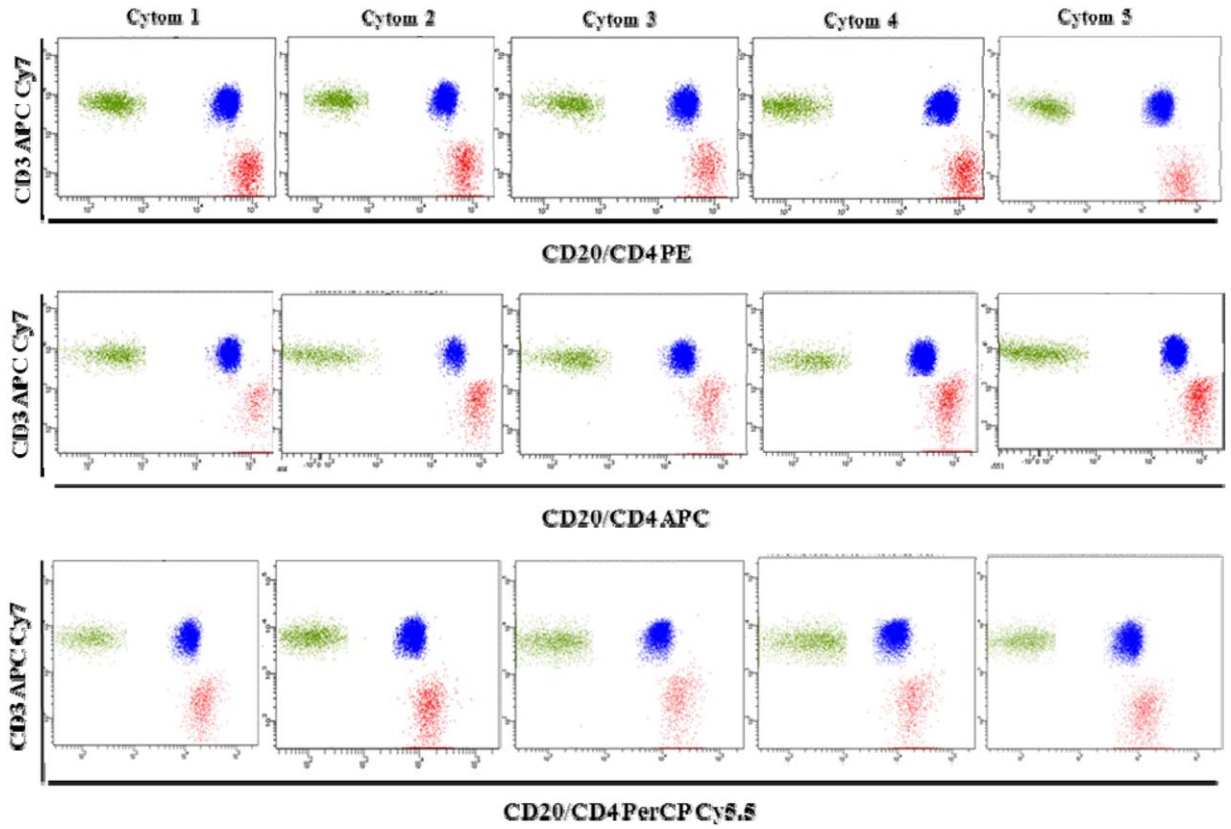

FIG. 3. Dot plots of CD20/CD4 vs. CD3 APC Cy7 for quantifying CD20 expression (red dots) relative to CD4 reference marker (blue dots) in three different fluorescence channels, PE, APC, or PerCP Cy5.5 on 5 different flow cytometer platforms. With the gating strategies illustrated in Figure 2, three distinct cell populations, CD20+ B cells (red), CD4+ T cells (blue), and CD4− T cells (green) are well separated for ABC value determination on the basis of Eq. (1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

variations associated with interfluorophore and interinstrument values provided in Table 2 are attributed to factors such as instrument difference and quality of antibody reagents.

The range of the ABC values from 68,080 to 117,840 from all three fluorochromes shows the variability of CD20 expression levels among the healthy individuals with no outlier identified by Grubbs' test at 95% confidence level, and is very similar to the range reported by Ginaldi and coworkers (28). In their study, direct immunofluorescence staining was performed with PE conjugated CD20 monoclonal antibody; microbeads with different binding capacities to mouse IgG were used to convert the MFI values to ABC values. The present ABC values are lower than the values we reported in 2006 (21) in which unimolar CD20 PE conjugate was used to determine the ABC values for CD20 expression according to QuantiBRITE™ PE bead calibration. It is likely that the molar ratio of PE and CD20 antibody for the off-the shelf CD20 PE conjugate used in this study is different compared with the unimolar CD20 PE conjugate we previously used. In such a case, unconjugated antibody may bind more strongly to the surface receptor and out-compete antibody labeled more than one PE resulting in lower MFI and lower CD20 ABC values due to higher expression level of CD20 than the level of CD4. Nonetheless, the use of off-the-shelf antibody reagents is cost effective and they are widely accessible and enable biomarker quantification in all fluorescence channels we tested.

SUMMARY

An ultimate goal of quantitative flow cytometry is to measure the number of antigens or ligand binding sites associated with a cell through the measurement of the number of antibodies bound per cell (ABC) (6–8). Specific receptors/antigens can serve as biomarkers for pathological conditions at various stages of a disease. Currently, there are no internationally recognized or validated biological cell reference standards with well characterized biomarker expression levels due to the complexity of producing such reference materials, that is, larger quantity, tight biomarker expression levels measured by two different techniques, resemblance of clinical samples, long term stability, and low cost storage conditions. While the development of biological cell reference materials are emerging (10,16,17,27), it is feasible to advance quantification schemes that take

Table 2
The ABC Values Obtained for CD20 Using Anti-CD20 (clone L27) on 10 Fixed Whole Blood Samples from AHDs Using Three Different Fluorophore Labels, PE, APC, and PerCP-Cy5.5 and Five Different Cytometer Platforms

	Cytom 1	Cytom 2	Cytom 3	Cytom 4	Cytom 5	Mean	CV%
ABC (CD20 PE)							
AHD-1	88,000	86,000	87,600	82,400	82,800	85,360	3.1%
AHD-2	89,600	99,200	97,600	94,800	100,400	96,320	4.5%
AHD-3	90,800	94,000	98,400	95,200	92,800	94,240	3.0%
AHD-4	90,400	88,400	93,600	92,000	91,600	91,200	2.1%
AHD-5	101,200	111,600	110,800	104,400	109,200	107,440	4.2%
AHD-6	120,000	116,800	117,600	115,200	116,800	117,280	1.5%
AHD-7	89,200	85,200	90,400	93,600	88,000	89,280	3.5%
AHD-8	69,200	64,800	68,400	63,600	74,400	68,080	6.2%
AHD-9	84,400	94,800	100,400	92,000	90,000	92,320	6.4%
AHD-10	85,200	83,200	85,200	87,200	82,000	84,560	2.4%
ABC (CD20 APC)[a]							
AHD-1	92,000	88,800	89,600	83,600	84,000	87,600	4.2%
AHD-2	93,200	95,200	98,800	96,800	93,600	95,520	2.4%
AHD-3	94,400	91,600	98,000	90,400	98,400	94,560	3.9%
AHD-4	94,400	96,000	97,200	90,000	95,600	94,640	2.9%
AHD-5	109,600	113,600	111,600	112,400	110,800	111,600	1.4%
AHD-7	96,000	94,800	94,800	86,800	87,600	92,000	4.9%
ABC (CD20 PerCP Cy5.5)							
AHD-1	84,400	90,800	93,200	89,600	87,200	89,040	3.8%
AHD-2	90,400	88,000	91,600	95,600	93,600	91,840	3.2%
AHD-3	92,400	92,400	100,400	99,200	92,000	95,280	4.4%
AHD-4	95,200	92,000	90,400	88,000	92,400	91,600	2.9%
AHD-5	104,800	96,000	100,800	107,200	112,000	104,160	5.9%
AHD-6	114,400	119,600	116,000	118,400	120,800	117,840	2.2%
AHD-7	92,000	89,200	97,600	100,000	87,200	93,200	5.8%
AHD-8	72,000	70,800	77,600	67,600	64,800	70,560	6.9%
AHD-9	89,200	89,200	95,200	86,800	85,600	89,200	4.1%
AHD-10	95,200	96,400	89,200	97,200	91,600	93,920	3.6%

[a]Measurements on the samples, AHD-6, AHD-8, AHD-9, and AHD-10, were not done.
The mean ABC value and CV are attained for each AHD from five different flow cytometers. The quantification of CD20 is carried out using Eq. (1) with a known CD4 expression level of ~40,000 for fixed whole blood (10,13,21,27).

advantage of the known expression level of CD4 on normal human T lymphocytes (10,12,13). To extend this approach to other cell types, autofluorescence of the reference cell type and the measured cell type should be taken into account.

In this study, quantification of CD20 on B lymphocytes relative to CD4 reference marker was carried out in two different assay formats, single tube assays and two separate tube assays. Our results demonstrated that relative biomarker quantification can be performed simultaneously and robustly using the single tube assay. With the use of the anti-CD20 antibody (clone L27) and anti-CD4 (clone SK3) in the single tube assay, the ABC values obtained for CD20 expression levels using three different fluorophore labels, PE, APC, and PerCP Cy5.5 were consistent over the five different instrument platforms for any given AHD. As shown in our previous studies (15,21), these results again provide evidence that supports the assumption that different antibodies against different antigens with the same fluorophore label can have similar average fluorescence per antibody values if they are produced by the same vendor. Additional variability such as sample age, anticoagulant used, and reference and test antibody clone selection are beyond the scope of this study.

To expand the scope of the investigation, flow cytometers from different manufacturers will be included in a future study to quantify several disease markers of CLL, that is, Zap-70, CD38, and CD20. Additionally, efforts on the production of lyophilized peripheral blood mononuclear cells (PBMC) prestained with fluorescently labeled anti-CD4 antibodies (clone SK3) would simplify the developed quantification scheme without the use of fresh human whole blood. This study demonstrates biomarker quantification can be performed simultaneously and robustly using the single tube assay relative to CD4 reference marker expression and multiple fluorochromes can be used to obtained comparable results.

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