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Flow Cytometer Performance Characterization, Standardization and Calibration against CD4 on T Lymphocytes Enables Quantification of Biomarker Expressions for Immunological Applications

Heba Degheidy[1]*, Steven Bauer[1], Gerald Marti[2], Lili Wang[3]*

[1]Center for Biologics Evaluation and Research, US Food and Drug Administration (FDA), Bethesda, Maryland, USA
[2]Center for Devices and Radiological Health, FDA, Silver Spring, Maryland, USA
[3]National Institute of Standards and Technology (NIST), Gaithersburg, Maryland, USA
Email: *lili.wang@nist.gov, *Heba.Degheidy@fda.hhs.gov

Abstract

There is an urgent need for developing a procedure for biomarker standardization and relative quantificationin clinical laboratories. Measuring the expression levels of cell antigens is critical for the diagnosis of many diseases, e.g. leukemia, lymphoma and immunodeficiency diseases. One of the most significant challenges in flow cytometry is obtaining inter-laboratory and intra-laboratory consistent and reproducible results across multiple cytometer platforms and locations longitudinally over time. To obtain measurement consistency, the target flow cytometer voltages should be optimized to segregate the negative population from the electronic noise, and to keep the brightest positive population within the dynamic range of each detector. Then target values should be determined and transferred to selected cytometers. In this study, we optimized a procedure for instrument standardization across three different flow cytometer platforms from the same vendor and in two different locations. The biomarker quantification was implemented on standardized instruments using CD4 expression on T lymphocytes with a known amount of antibody bound per cell as a quantification standard. Our results on blood cell subset typing and CD19 quantification demonstrated that consistent and reliable results could be accomplished between instruments using the developed procedure. Quantitating the expression levels of certain cell biomarkers relative to a known reference marker before, during, and after therapy would provide

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*Corresponding authors.

important information for monitoring antibody-based therapy and could be potentially used to adjust dosing. Presently, we are implementing this protocol to quantify critical disease biomarkers, and making necessary modifications to the procedure to include instruments from different instrument manufacturers.

Keywords

Flow Cytometry, Biomarker Quantification, Instrument Standardization, Target Median Fluorescence Intensity (MFI)

1. Introduction

Flow cytometry allows simultaneous measurement of the light scatter properties of different cell types as well as the fluorescence intensities of distinct fluorophores attached to these cell types. It is a powerful analytical tool that has been widely used to perform immunophenotyping, cell counting and numerous cell function assessments, such as intracellular cytokine production, protein phosphorylation, cell proliferation and apoptosis.

Development of quantitative flow cytometry (QFC) has been urged for clinical biomarker standardization because accurate and reproducible results will greatly aid our understanding of diseases, enable timely diagnosis and allow proactive monitoring of therapeutic and off-target drug side effects. Standardization of flow cytometryin hematological malignancies has been a great challenge. A few published studies describe standardization of data at the analytical stage by exchanging the list mode files to harmonize and standardize the analysis of minimal residual disease in multiple study groups [1]-[4]. Letestuand co-workerse valuated ZAP-70 expression in Chronic Lymphocytic Leukemia (CLL) in a multicenter international harmonization process [5]. Kraan *et al.* [6] presented general rules for cytometer setup using up to four colors in clinical settings using analog flow-cytometric systems, and Shankey *et al.* [7] reported complete standardization of 4-color ZAP-70 investigation among three institutions. Additionally, the Euro Flow Consortium demonstrated successful standardization of flow cytometer settings and measurement protocols for immunophenotyping in a multicenter study [8].

Quantitative flow cytometry has been demonstrated in a single laboratory study [9]-[11]. However, multicenter quantitative studies including various flow cytometer platforms have not yet been reported, suggesting the enormous difficulty of the task. This type of study requires both multi-platform instrument standardization and the use of a biological reference marker with a known expression level for ultimate biomarker quantification. The instrument standardization includes multiple steps, e.g. characterization of each instrument performance, target instrument identification, optimization of the target instrument fluorescence detector voltages as well as the application of the median fluorescence intensity (MFI) values from the target instrument to other instrument platforms. Once all flow cytometers are standardized, cell biomarker expression levels can be quantified with a reference biomarker, e.g. CD4 on normal human T lymphocytes with a known number of the antibody bound per cell (ABC) value [12]-[14]. In the present study, we have developed an instrument standardization procedure across three different flow cytometer platforms from the same vendor with somewhat different optical configurations and in two different locations. After the standardization of these instruments, CD19 quantification is carried out to validate the ABC quantification approach and demonstrate that consistent and reliable results can be accomplished between instruments using the developed procedure.

2. Material & Method

Four flow cytometry instruments from the same manufacturers in two different laboratories were used in this study. Instrument 1 (FACSCanto II), Instrument 2 (LSRFortessa), Instrument 3 (FACS Aria III) and Instrument 4 (FACSAria II) are all from BD Biosciences (San Jose, CA). The first three Instruments are located at FDA and the fourth instrument is located at NIST. For this study all four instruments were configured with three lasers: blue (488 nm), red (633 or 640 nm) and violet (405 or 407 nm) lasers (**Table 1(a)**). These instruments have flexible and compatible optical configurations with the exceptions described in **Table 1(b)**, which could potentially be used in diagnostic laboratories. Cytometer Setup & Tracking (CS & T) beads were from BD Biosciences; they are composed of equal concentrations of dim, mid, and bright dye embedded polystryrene beads [2]

Table 1. (a) Laser excitation wavelength for instruments used in the study; (b) Optical configuration and filter specifications for instruments used in this study.

(a)

	Laser Excitation Wavelength		
	Blue	**RED**	**Violet**
Cytometer (1)	488	633	405
Cytometer (2)	488	640	405
Cytometer (3)	488	633	407
Cytometer (4)	488	633	405

(b)

	Fluorescence Channels							
	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7	HV450	HV500
Cytometer (1)	502 LP 530/30 BP	556 LP 585/42 BP	655 LP 670 LP	735 LP 780/60 BP	660/20 BP	735 LP 780/60 BP	450/50 BP	502 LP 510/50 BP
Cytometer (2)	505 LP 530/30 BP	550 LP 575/26 BP	685 LP 695/40 BP	750 LP 780/60 BP	670/14 BP	750 LP 780/60 BP	450/50 BP	475 LP 525/50 BP
Cytometer (3)	502 LP 530/30 BP	556 LP 585/42 BP	655 LP 695/40 PB	735 LP 780/60 BP	660/20 BP	735 LP 780/60 BP	450/40 BP	502 LP 510/50 BP
Cytometer (4)	502 LP 530/30 BP	556 LP 585/42 BP	685 LP 695/40 BP	735 LP 780/60 BP	670/14 BP	735 LP 780/60 BP	450/40 BP	502 LP 530/30 BP

μm (dim bead), 3 μm (mid and bright beads)]. The same master lot of CS & T beads was used throughout the study to define the base line for each instrument and daily QC/QA for the instrument. In addition, the bright bead population wasutilized to determine the target MFI values that were transferred to the other flow cytometers. Ultra Rainbow bead kit (Catalog #: URCP-38-2K) containing a blank and five different fluorescence intensity populations was acquired from Spherotech Inc (Lake Forest, IL) and used to calibrate the linearity of the fluorescence channels used for biomarker quantification in the unit of equivalent reference fluorophore fluorescence (ERF) [10].

Anonymous heparinized normal donor samples were obtained from NIH's Department of Transfusion Medicine according to the institutional review boards (IRB) guidelines. The unstained normal donor blood was used to adjust the Photomultiplier tube (PMT) voltage for each fluorescence detector to adequately segregate the auto-fluorescent negative signal from instrument electronic noise in such a way that dim fluorescent events can be discriminated from the true negative. Singly stained normal donor blood was used to ensure that the brightest populations are within the dynamic scale of each detector and to derive the compensation matrix. Lastly, multicolor stained normal donor blood was employed to compare blood cell subset typing results across the selected platforms and determine CD19 expression levels on B lymphocytes.

3. Results & Discussion

3.1. Selection of a Target Flow Cytometer

A two-step approach was used for the selection of a target cytometer [15]: 1) Forall cytometers to be standardized, a baseline performance was carried out for instrument performance characterization. The robust standard deviation of electronic noise (rSD$_{EN}$), linearity maximum (Lin Max), and linearity minimum (Lin Min) values were determined for each detector as provided from the baseline performance report; 2) A target cytometer was determined across multiple platforms based on the highest rSD$_{EN}$ and lowest Lin Max for each detector (**Table 2**). On the basis of these two criteria, Cytometer (1) was identified as the primary target cytometer for this study.

3.2. Photomultiplier (PMT) Voltage Adjustment

An unstained normal donor sample was first used to adjust PMT voltage for each fluorescence detector. Briefly, 10 ml of heparinized whole blood was washed twice with phosphate buffer saline (PBS; pH 7.4) containing 5% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St Louis, MO). The sample was then lysed using 1X FACS Lysing

Table 2. Cytometer characterization based on electronic noise robust standard deviation (rSD$_{EN}$) and linearity maximum (Lin Max).

		FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7	V450	V500
Cytometer (1)	Lin Max	240376	238463	231651	230373	229919	234132	242158	232150
	rSD$_{EN}$	23.9	23.9	23.9	24	20.2	21.2	20.1	21
Cytometer (2)	Lin Max	237383	244257	225525	233823	238165	237708	242866	237772
	rSD$_{EN}$	13.1	15.3	13.6	13.7	15	13.9	13.2	13.2
Cytometer (3)	Lin Max	237773	235772	237051	231981	237997	240282	236451	238260
	rSD$_{EN}$	8.6	8.6	8.6	8.6	7.3	7.3	5.4	5.5
Cytometer (4)	Lin Max	236294	234214	233874	225073	230910	237487	239505	237028
	rSD$_{EN}$	12.1	12.0	16	14	10.8	10.8	9.1	9.1

Solution (BD Biosciences) according to the manufacturer's recommendations. After centrifugation, the cell pellet was washed twice with 5% FBS/PBS and re-suspended in 2 ml of the same buffer. On the flow cytometers, the FSC/SSC gate for lymphocytes was used and the PMT voltages of the primary target instrument were adjusted to established voltages in such a way that the robust standard deviations of the autofluorescence signals from unstained cells are within 2.5 to 3 times the rSD$_{EN}$ of each detector to be used in the analysis. These optimized PMT voltages were further verified using singly stained blood control stained with either CD45 or CD3for each channel to ensure that the brightest populations are within the dynamic scale of each detector. In summary, the PMT voltages were adjusted so that negatively stained cell populations were located above the electronic noise on the low end, and the positively stained cell populations were within the linear range of the detector for each fluorochrome used.

3.3. Target Values Determination and Application for Multiplatform Standardization

Using the optimized PMT values obtained from the previous step, the bright CS & T bead population was gated and its fluorescence intensities were recorded in all channels of the primary target flow cytometer. These intensity values were sed as preliminary "Target MFIs" (See **Supplement Figure S1**) and transferred to all other companion instruments utilized in this study. After each companion cytometer passed its routine CS & T performance check, the PMT voltages in these instruments were adjusted in such way that the same bright bead population has equal or nearly equal "Target MFI" in each detector. In other words, the bright bead MFI values were copied from the primary instrument to other cytometers to be standardized by adjusting PMT voltages to bring the same bright bead peak to the target MFI of each detector. The adjusted PMT voltages of all instruments were recorded and followed on a daily basis to monitor instrument stability. With the recorded PMT voltages applied, the MFI of the bright CS & T bead population in each fluorescence channel of any cytometer used in the study was monitored on the day of use with the corresponding target MFIs for that instrument after the instrument standardization process was completed. Variations of only a few volts were acceptable. Whenever instrument performance failed on any instrument, measures such as thorough cleaning, de-gassing flow cell and laser delay verification were taken. When the performance was not restored to pass the monitoring criteria, a service visit was requested. Note that if the target MFI setting resulted in suboptimal PMT setting on any instrument, the target MFI values were adjusted accordingly on the target flow cytometer until consensus target MFI values were reached under optimal PMT settings for all instruments.

3.4. Verification of Instrument Standardization by Single Tube Analysis of Blood Cell Subsets

Sample Staining Procedure: To validate the cytometer standardization procedure and ensure data comparability across all four cytometers, a one ml of heparinized whole blood samples was used at each site within 6 - 8 hours after venipuncture. Prior to staining, the whole blood was washed twice with phosphate buffer saline (PBS; pH 7.4) containing 5% FBS. The sample was then stained in one location with the following seven reagents: CD20 eFluor 450 (eBiosciences, San Diego, CA), CD45 V500, CD3 APC-Cy7, CD5 PE-Cy7, CD4 PE, CD8 APC, CD19 PerCP-Cy5.5 (BD Biosciences) for 30 min in the dark. After staining, the cells were washed twice and lysed using 1X FACS Lysing Solution. After one wash, cells were fixed in 1% paraformaldehyde and ac-

quired within 1 - 2 hours of cell fixation at both locations. Unstained control cells and singly stained tubes for the eight fluorochromes (FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7, eFluor 450 and V500) were also prepared and used to set the flow cytometer compensation. The number of events collected in each sample of these experiments varied from 500,000 to 1 million events.

Blood Cell Subset Typing: The stained normal donor sample was acquired in the four optimized and standardized flow cytometers at a "low" flow rate mode within the first 2 hour after sample preparation. Data were subsequently analyzed using the FACSDiva V6software. A doublet exclusion gate based upon (FSC-A vs. FSC-H) was utilized to gate on singlet cells, and within this singlet cell gate, a lymphocyte gate was drawn in a dot plot of FSC-A vs. SSC-A (**Supplement Figure S2**). Using a dot plot of CD45 vs. SSC-A under the lymphocyte gate, aR1 gate was drawn around the CD45 positive cells and used for all subsequent gating. **Figure 1** shows comparative dot plots of CD3 vs. CD20 and CD20 vs. CD5 and **Figure 2** displays dot plots of CD45 vs. CD3 and CD45 vs. CD4, both under R1 gate. The percentage of CD3, CD4, CD8, CD19, and CD20 were obtained and provided in **Table 3**.

Under the CD45+ cell gate, the percentage of CD3 positive cells was 53.8% for instrument 1, 53.1% for instrument 2, 53.7% for instrument 3, and 53.3% for instrument 4, respectively. Under the same CD45+ main gate, the percentage of CD4 positive cells was 33% for instrument 1, 32.7% for instrument 2, 32.1% for instrument 3, and 32.6% for instrument 4, respectively. For CD8 expression, the percentage obtained was 22.2%, 19.4%, 20%, and 20.6% for instruments 1 to 4, respectively. The percentage of CD19 positive cells was 29.6%, 31.7%, 32.5%, and 30% for instruments 1 to 4, respectively. Lastly, for cells expressing CD20 the percentage obtained was 28.2%, 31.8%, 28.9%, and 31.5% for instruments 1 to 4, respectively.

When gated on CD3 positive cells, the percentage of CD4+ cells was 53.3%, 58%, 58.8%, and 57.6% for instruments 1 to 4, respectively; for cells with CD 8 expression the percentage obtained was 35.5%, 34.9%, 35%, and 36.7% for instruments 1 to 4, respectively. The results on the percentages of the blood cell subsets are reasonably consistent among the four instruments demonstrate that these instruments are standardized with the procedure implemented. The slightly larger coefficient variations of the mean percentages derived for CD8+, CD19+ and CD20+ cells under the CD45+ cell gate (**Table 3**) might be related to difference in band path pass filters equipped, e.g. in PerCP Cy5.5 and APC channels (**Table 1(b)**), and wider expression of CD45 on many different blood cell types.

3.5. Biomarker Quantification

Quantification Scheme: Three different flow cytometer platforms (instrument 1 to 3) at one location were used for CD19 quantification, a B lymphocyte marker after the three instruments were standardized. The quantification scheme using CD19 APC-Cy7 as an example is illustrated in **Figure 3**. The linearity of APC-Cy7 channel is assured by using Ultra Rainbow beads for linearity calibration in the unit of the equivalent APC fluorescence (EAF) [10] (square symbol). Using CD4 APC-Cy7 as a reference biomarker with a known antibody bound per cell (ABC) value of 45,000 for fixed whole blood [14], the linear fluorescence calibration scale (left Y-axis in **Figure 3**) obtained by using Rainbow beads is transformed to the ABC scale (right Y-axis). The quantification of CD19 APC-Cy7 is then carried out using the converted ABC calibration on the right Y-axis. With this relative quantification scheme, it is assumed that different antibodies against different antigens have very similar average fluorescence per antibody values because both CD4 APC-Cy7 and CD19 APC-Cy7 are from the same vendor such that the same conjugation chemistry and process control for APC-Cy7 labeling were applied. It is critical that the labeled antibodies are free of unconjugated APC-Cy7 fluorophores due to likely higher binding affinity for unconjugated antibodies than fluorophore conjugated antibodies. This assumption is further tested in this study using different fluorophore labeled antibodies, CD4 PerCP-Cy5.5 and CD19 PerCP-Cy5.5. Note that monoclonal antibody affinity issue on both CD4 reference marker and the marker of interest has not been taken into account. However, high affinity monoclonal antibodies against specific antigen epitopes are generally used for quantitative flow cytometry measurements in terms of ABC values.

Quantification of CD19 Expression: Four tubes were stained with either CD19 PerCP-Cy5.5, CD4 PerCP-Cy5.5, CD19 APC-Cy7 or CD4 APC-Cy7 together with CD45 V500. A doublet exclusion gate based upon (FSC-A vs. FSC-H) was utilized to gate on only singlet cells (**Supplement Figure S3**). Within this singlet cell gate, a lymphocyte gate was drawn in a dot plot of FSC-A vs. SSC-A. Under the lymphocyte gate a dot plot of CD45 vs. SSC-A were used to gate on CD45 positive cells and used for all subsequent gating. Dot plot of
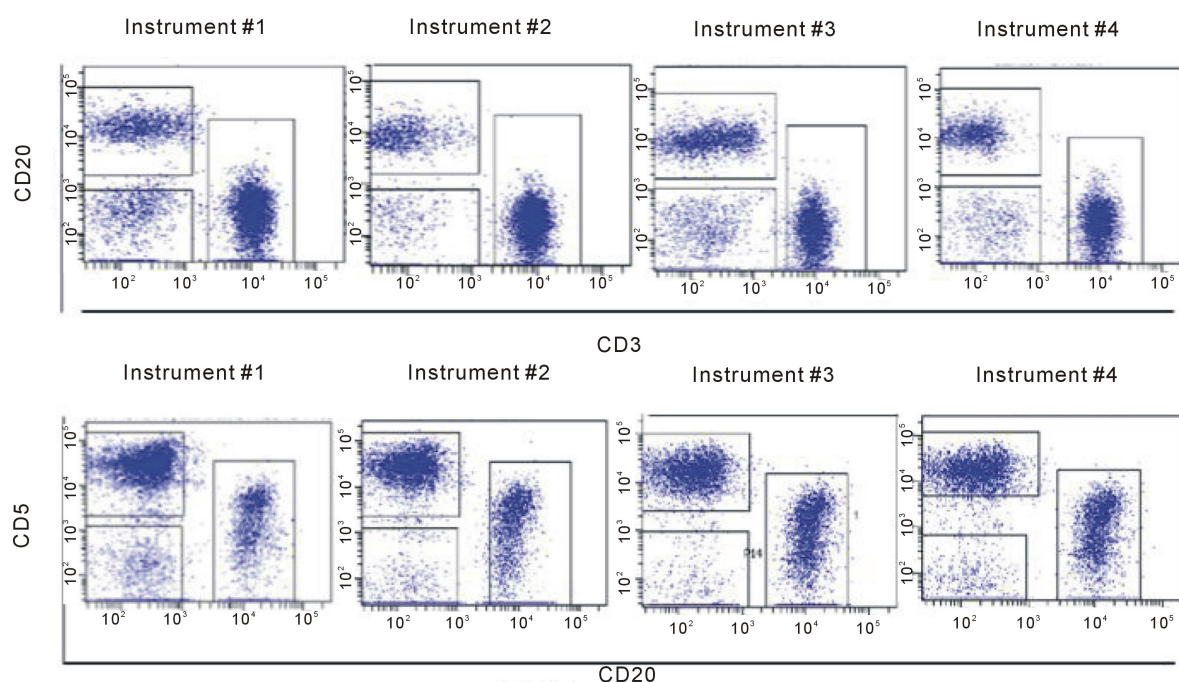
Figure 1. Comparative Dot plots of CD3 on X-axis vs. CD20 on Y-axis upper row and CD20 on X-axis vs. CD5 on Y-axis lower row for one normal donor. Data was obtained from the same sample acquired on four different flow cytometersin two different locations. The instruments were set using one set of target values as described in "Method". Percentages of positive cells were determined after gating on CD45 positive cells following the doublet exclusion.
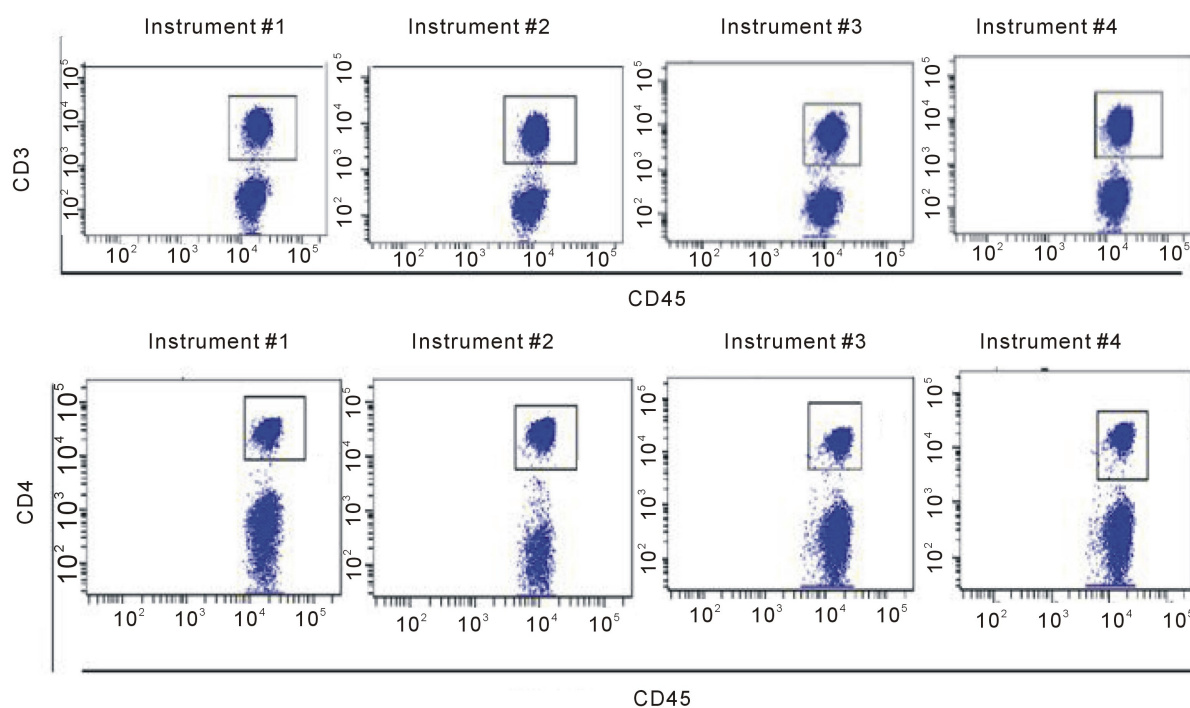

Figure 2. Comparative Dot plots of CD45 on X-axis vs. CD3 on Y-axis upper row and CD45 on X-axis vs. CD4 on Y-axis lower row for one normal donor. Data was obtained from the same sample acquired on four different flow cytometersin two different locations. The instruments were set using one set of target values as described. Percentages of positive cells were determined after gating on CD45 positive cells following doublet exclusion.
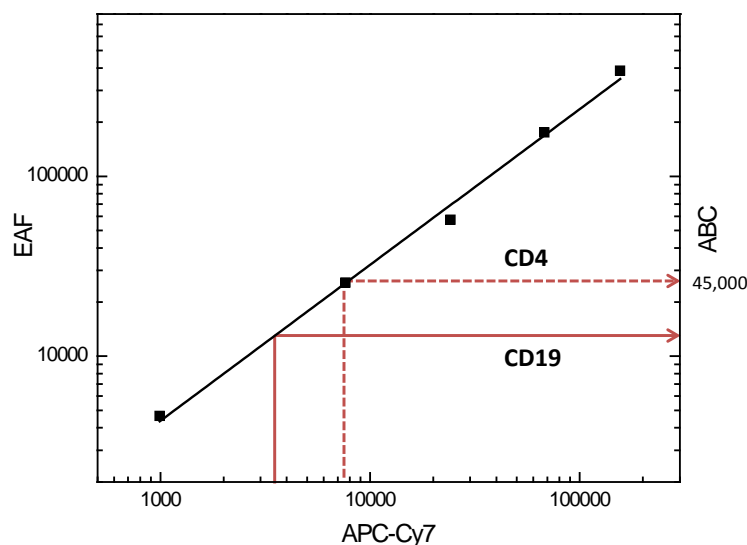
Figure 3. Schematic of CD19 quantification on APC-Cy7 channel. The linearity of APC-Cy7 channel is assured by using Ultra Rainbow beads for the calibration in the unit of the equivalent APC fluorescence (EAF) [10] (square symbol). The adjusted R-squared for the linear fit to the log transformed data as shown is 0.994. Using CD4 APC-Cy7 as a reference biomarker with a known ABC value of 45,000 for fixed whole blood, the CD19 expression level in ABC unit is determined.

Table 3. Percentage of CD3, CD4, CD8, CD19, and CD20 cells gated on CD45 positive cells obtained from individual instruments, and mean percentage and associated CV of each cell subset averaged from all four instruments.

	Gated on CD45+ cells					Gated on CD3+ cells	
	CD3	CD4	CD8	CD19	CD20	CD4	CD8
Instrument 1	53.8	33.0	22.2	29.6	28.2	58.3	35.5
Instrument 2	53.1	32.7	19.4	31.7	31.8	58.0	34.9
Instrument 3	53.7	32.1	20	32.5	28.9	58.8	35
Instrument 4	53.3	32.6	20.6	30.0	31.5	57.6	36.7
Mean (CV%)	53.5 (0.6)	32.6 (1.1)	20.6 (5.8)	31.0 (4.4)	30.1 (6.0)	58.2 (0.9)	35.5 (2.3)

CD45 vs. CD 19 or CD45 vs. CD 4 was used to gate on either CD19+ cells or CD4+ cells, respectively, using either APC-Cy7 or PerCP-Cy5.5 fluorochrome.

The median fluorescence intensity (MFI) values for CD19 receptor protein in both APC-Cy7 and PerCP-Cy5.5 channels on two fixed normal whole blood samples are provided in **Table 4**. Although these values were obtained on the same day with assured linear responses from both fluorescence channels of the standardized instruments, they show large variations with at least 20% of CV for the same sample among different instruments. Upon the application of the ABC calibration with CD4 as the reference biomarker, an ABC value for CD19 APC-Cy7 for the sample, ND-1 and instrument 2 is calculated to be

$$ABC_{CD19} = \frac{MFI_{CD19}}{MFI_{CD4}} \times ABC_{CD4} = \frac{3746}{7534} \times 45000 = 22374,$$

with the assumption that the effective fluorescence per antibody for anti-CD4 and anti-CD19 made by the same manufacturer is the same [16]. The ABC values obtained for CD19 as summarized in **Table 5** are highly comparable for the same donor sample, independent of fluorochrome label (APC-Cy7 or PerCP-Cy5.5) and cytometer plat form used. The mean ABC value and associated CV summarized over three instruments and two fluorophores are 22,153 and 2.8% for ND-1 and 25,519 and 4.6% for ND-2, respectively. The consistent results from two different fluorophore labels verify the validity of the assumption that different antibodies against different antigens with the same fluorophore label likely have very similar average fluorescence per antibody values as long as they are produced by the same vendor under rigorous good manufacturing practice (GMP). The study results demonstrate comparable and consistent biomarker quantification across different fluorescence channels

Table 4. Median fluorescence intensity (MFI) values for CD19 in APC-Cy7 and PerCP-Cy5.5 channels obtained from two normal donors (ND). The mean MFI value and CV (column 5 and 6) are obtained for each donor from three different instruments.

	MFI (CD19 APC-Cy7)				
	Instrument 1	Instrument 2	Instrument 3	Mean	CV%
ND-1	2588	3746	2671	3002	21.5
ND-2	2841	4280	2960	3360	23.8
	MFI (CD19 PerCP-Cy5.5)				
	Instrument 1	Instrument 2	Instrument 3	Mean	CV%
ND-1	3913	2763	2588	3088	23.3
ND-2	4303	3176	3021	3500	20.0

Table 5. The antibody bound per cell (ABC) values obtained for CD19 on two fixed normal whole blood samples using two different fluorophore labels, APC-Cy7 and PerCP-Cy5.5 and three different cytometer platforms. The mean ABC value and CV (column 5 and 6) are obtained for each donor from three different instrument platforms. The quantification of CD19 is carried out relative to CD4 with a known expression level of 45,000 for fixed whole blood.

	ABC (CD19 APC-Cy7)				
	Instrument 1	Instrument 2	Instrument 3	Mean	CV%
ND-1	22,136	22,374	21,913	22,141	1.0
ND-2	24,819	25,950	25,256	25,342	2.3
	ABC (CD19 PerCP-Cy5.5)				
	Instrument 1	Instrument 2	Instrument 3	Mean	CV%
ND-1	21,406	23,240	21,850	22,165	4.3
ND-2	23,826	27,316	25,944	25,695	9.7

and cytometer platforms can be only achieved with the use of the ABC calibration scheme.

4. Summary

Quantitative flow cytometer assays are routinely used in clinical laboratories to estimate the number of specific receptor/antigen sites on the surface or inside of cells taken from blood, lymph nodes, and bone marrow. It is also appropriate for other body fluids such as a bronchial lavage, pleural and peritoneal fluids and even bladder washings. Specific receptors/antigens can serve as biomarkers for pathological conditions at various stages of a disease. Additionally, flow cytometry measurements are used to support many clinical, pharmacologic, and calibration and measurement capability claims used for drug/vaccine, device, and biologics product development and approval. However, it is still a great challenge to obtain comparable results from different laboratories using different instrument platforms due to the lack of the optimized and standardized procedures and biological reference materials. This study was designed in a small scale to develop a standardized procedure for quantifying CD19 expression level relative to CD4 reference marker. The comparable results of CD19 expression from two normal individuals 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. To expand the scope of the investigation, flow cytometers from different manufacturers are included in an on-going study to quantify several disease markers of CLL. In addition, efforts on the production of lyophilized peripheral blood mononuclear cells (PBMC) pre-stained with fluorescently labeled anti-CD4 antibodies would simplify the developed quantification scheme without the use of fresh human whole blood.

Acknowledgements

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Abbreviations

FITC, fluorescein isothiocyanate;
PE, phycoerythrin;
PerCP-Cy5.5, peridinin-chlorophyll protein-cyanine 5.5;
PE-Cy7, phycoerythrin-cyanine 7;
APC, allophycocyanin;
APC-Cy7, allophycocyanin-cyanine 7;
HV450, Horizon V450;
HV500, Horizon V500;
LP, long pass filter;
BP, band pass filter;
CS & T beads, cytometer setting & tracking beads;
MFI, Median Fluorescence Intensity;
rSD$_{EN}$, electronic noise robust standard deviation;
Lin Max, linearity maximum;
ND, normal donor;
ABC, antibody bound per cell;
FDA, Food and Drug Administration;
NIST, National Institute of Standards and Technology.

Supplement
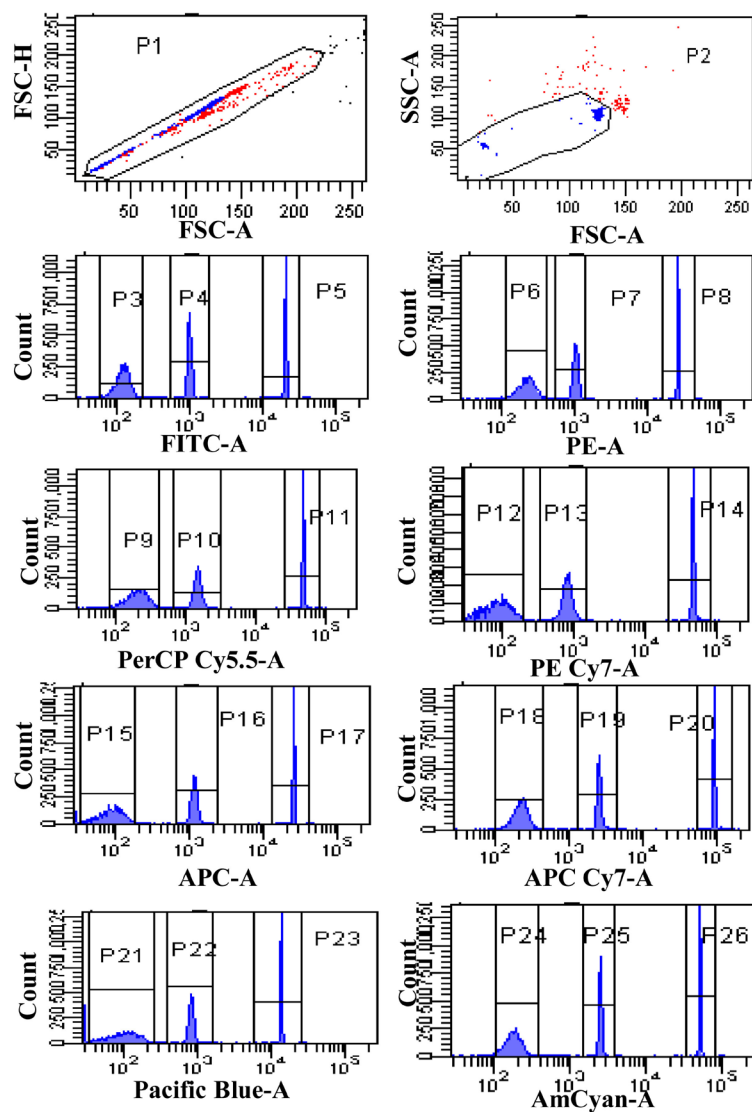
Population	FITC Mean	PE Mean	PerCP-Cy5.5 Mean	PE-Cy7 Mean	APC Mean	APC-Cy7 Mean	Pacific Blue Mean	AmCyan Mean
P3	180	205	210	44	273	274	109	200
P4	1324	823	1408	512	1982	3035	1094	2871
P5	<u>33,273</u>	25,903	39,391	25,034	31,579	83,303	19,983	69,327
P6	175	210	202	44	274	276	108	200
P7	1318	824	1407	511	1977	3030	1092	2866
P8	33,274	<u>25,905</u>	39,395	25,042	31,581	83,332	19,975	69,327
P9	175	202	213	39	273	273	105	199
P10	1318	821	1405	505	1980	3009	1095	2869
P11	33,275	25,906	<u>39,389</u>	25,032	31,588	83,310	19,976	69,330
P12	417	341	457	101	614	774	345	765
P13	1300	810	1376	586	1948	2987	1078	2822
P14	33,215	25,909	39,592	<u>25,376</u>	31,382	84,446	19,990	69,393
P15	177	205	210	46	286	270	116	205

Continued

P16	1321	824	1407	511	1992	3035	1093	2873
P17	33,230	25,890	39,463	25,179	<u>31,458</u>	83,736	19,969	69,305
P18	177	206	202	42	271	288	107	202
P19	1331	831	1408	508	1990	3027	1111	2897
P20	33,177	25,878	39,585	25,421	31,334	<u>84,447</u>	19,968	69,322
P21	176	205	215	44	273	273	113	196
P22	1320	823	1404	512	1982	3036	1094	2872
P23	33,277	25,908	39,402	25,051	31,582	83,366	<u>19,972</u>	69,334
P24	175	204	206	45	274	273	104	203
P25	1320	823	1405	512	1981	3033	1094	2871
P26	33,270	25,900	39,384	25,024	31,573	83,274	19,990	<u>69,322</u>

Figure S1. Mean Fluorescent Intensity (MFI) values of cytometer setting & tracking (CS & T) beads on the target flow cytometer were obtained using FACS Diva software. After gating on the singlet's using FSC-A vs. FSC-H, a subsequent gate on the two size beads using SSC-A vs. FSC-A was performed. The negative, dim and bright bead populations were gated in a histogram for each channel. The target MFIs for the eight fluorescence channels of the target flow cytometer are the MFIs of the bright CS & T bead population marked in the statistical review table on the right.The CVs for the bright CS & T bead population over the eight fluorescence channels are generally no more than 3%.
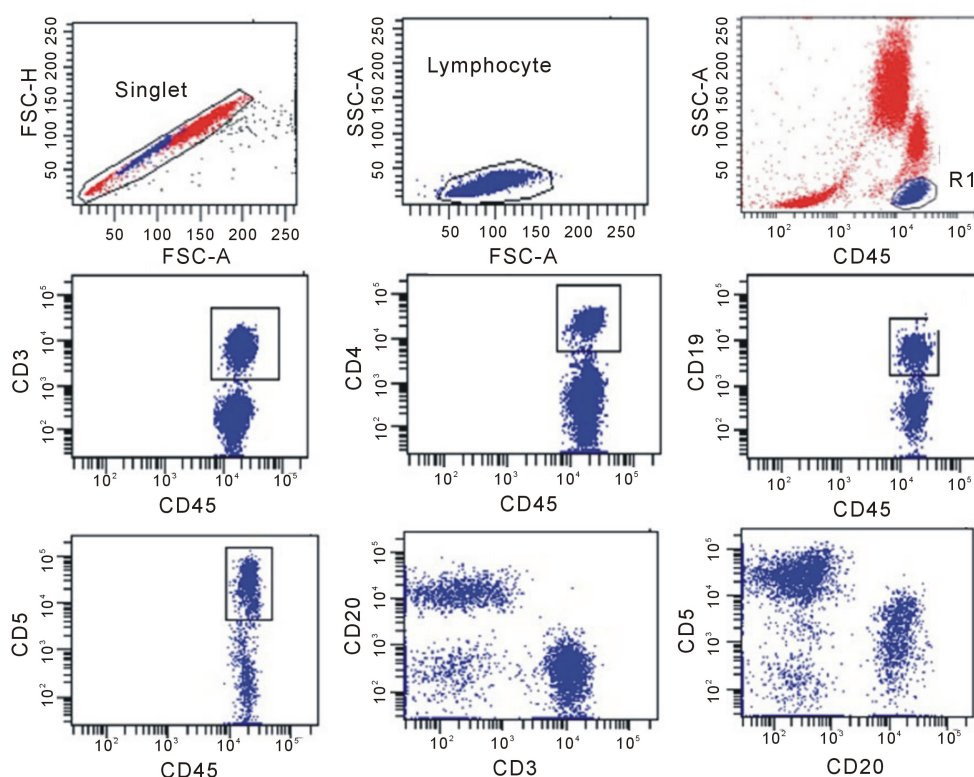

Figure S2. Gating Strategy for percentage of positive cell subset typing. A doublet exclusion gate based upon (FSC-A vs. FSC-H) was utilized to gate on singlet cells (upper left dot plot), and within this singlet cell gate, a lymphocyte gate was drawn in a dot plot of FSC-A vs. SSC-A (Upper middle dot plot). Using a dot plot of CD45 vs. SSC-A underthe lymphocyte gate, R1 gate was drawn around the CD45 positive cells and used for all subsequent gating (dot plots of CD45 vs. CD3, CD45 Vs. CD4, CD45 vs. CD19, CD45 vs.CD5, CD3 vs. CD20 and CD20 vs. CD5 were shown).
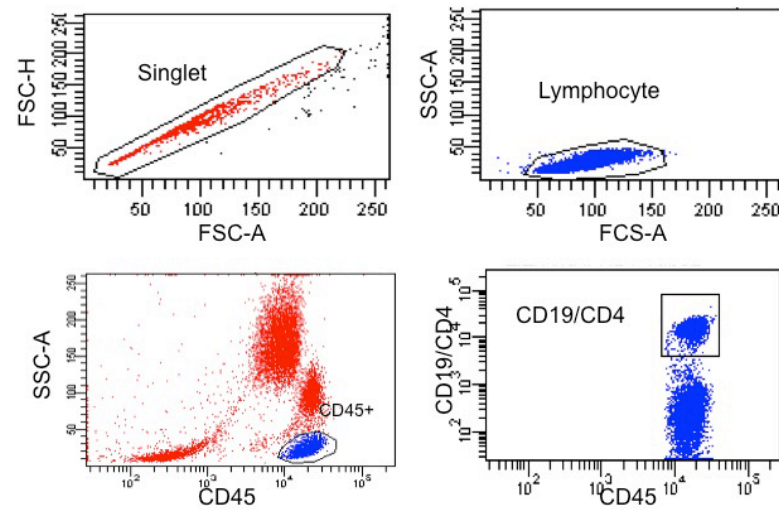
Figure S3. Gating Strategy for CD19 Quantification.A doublet exclusion gate based upon (FSC-A vs. FSC-H) was utilized to gate on singlet cells (upper left dot plot), and within this singlet cell gate a lymphocyte gate was drawn in a dot plot of FSC-A vs. SSC-A (Upper right dot plot). Using a dot plot of CD45 vs. SSC-A underthe lymphocyte gate, R1 gate was drawn around the CD45 positive cells (lower left dot plot) and used for subsequent gating. The lower right combined dot plot of CD45 vs. CD19/CD4 shows either CD19 or CD4 gate for CD19 quantification relative to CD4 reference biomarker.

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