ORIGINAL ARTICLE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Quantification of Cells with Specific Phenotypes II: Determination of CD4 Expression Level on Reconstituted Lyophilized Human PBMC Labelled with Anti-CD4 FITC Antibody

L. Wang,[1*] R. Stebbings,[2] A. K. Gaigalas,[1] J. Sutherland,[2] M. Kammel,[3] M. John,[3] B. Roemer,[4] M. Kuhne,[5] R. J. Schneider,[5] M. Braun,[6] A. Engel,[7] D. Dikshit,[8] F. Abbasi,[9] G. E. Marti,[9] M. Sassi,[10] L. Revel,[10] S. K. Kim,[11] M. Baradez,[12] T. Lekishvili,[12] D. Marshall,[12] L. Whitby,[13] W. Jing,[14] V. Ost,[15] M. Vonsky,[16] J. Neukammer[3]

[1]Biosystems and Biomaterials Division, NIST (National Institute of Standards and Technology), Gaithersburg, Maryland 20899

[2]Biotherapeutics Group, NIBSC (National Institute for Biological Standards and Control), Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom

[3]Division of Medical Physics and Metrological Information Technology, PTB (Physikalisch-Technische Bundesanstalt), Berlin 10587, Germany

[4]Abbott GmbH & Co. KG, Germany

[5]Department of Analytical Chemistry, BAM Federal Institute for Materials Research and Testing, Berlin, D-12489, Germany

[6]Beckman Coulter GmbH, Europark Fichtenhain B13, Krefeld 47807, Germany

[7]Becton Dickinson, Tullastraße 8-12, Heidelberg 69126, Germany

[8]Medicinal and Process Chemistry Division, CDRI (Central Drug Research Institute), Chattar Manzil Palace, Mahatma Gandhi Marg, Lucknow, Uttar Pradesh 226001, India

[9]CDRH/FDA (Center for Devices and Radiologic Health Food and Drug Administration), Bethesda, Maryland 20892

Abstract

This report focuses on the characterization of CD4 expression level in terms of equivalent number of reference fluorophores (*ERF*). Twelve different flow cytometer platforms across sixteen laboratories were utilized in this study. As a first step the participants were asked to calibrate the fluorescein isothiocyanate (FITC) channel of each flow cytometer using commercially available calibration standard consisting of five populations of microspheres. Each population had an assigned value of equivalent fluorescein fluorophores (*EFF* denotes a special case of the generic term *ERF* with FITC as the reference fluorophore). The *EFF* values were assigned at the National Institute of Standards and Technology (NIST). A surface-labelled lyophilized cell preparation was provided by the National Institute of Biological Standards and Control (NIBSC), using human peripheral blood mononuclear cells (PBMC) pre-labeled with a FITC conjugated anti-CD4 monoclonal antibody. Three PBMC sample vials, provided to each participant, were used for the CD4 expression analysis. The PBMC are purported to have a fixed number of surface CD4 receptors. On the basis of the microsphere calibration, the *EFF* value of the PBMC samples was measured to characterize the population average CD4 expression level of the PBMC preparations. Both the results of data analysis performed by each participant and the results of centralized analysis of all participants' raw data are reported. Centralized analysis gave a mean *EFF* value of 22,300 and an uncertainty of 750, corresponding to 3.3% (level of confidence 68%) of the mean *EFF* value. The next step will entail the measurement of the *ERF* values of the lyophilized PBMC stained with labels for other fluorescence channels. The ultimate goal is to show that lyophilized PBMC is a suitable biological reference cell material for multicolor flow cytometry and that it can be used to present multicolor flow cytometry measurements in terms of ABC (antibodies bound per cell) units.

Key terms

surface labelled lyophilized PBMC; CD4 expression level; FITC; equivalent fluorescein fluorophore (*EFF*); quantitative flow cytometry; calibration; standard measurement procedure; measurement uncertainty; reference cell material

Cluster of differentiation 4 (CD4) is a glycoprotein on the surface of T-helper cells, monocytes, macrophages and dendritic cells. As a co-receptor, CD4 amplifies the signal generated by the T cell receptor, which is essential for activation of many molecules involved in the signalling cascade of an activated T cell. The CD4-positive

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[10]Amount of Substance, INRIM (Istituto Nazionale Di Ricerca Metrologica), Strada Delle Cacce 91, Torino 10135, Italy

[11]Bioanalysis, KRISS (Korea Research Institute of Standards and Science), Doryong-Dong, Yuseong-Gu, Daejeon 305-340, Korea

[12]Science and Innovation, LGC Limited, Teddington, Middlesex, TW11 0LY, United Kingdom

[13]UK NEQAS (UK National External Quality Assessment Service), Sheffield, South Yorkshire S10 2QD, United Kingdom

[14]Division of Medical and Biological Measurement, NIM (National Institute of Metrology), No 18, Bei San Huan Zhong Lu, Beijing, China

[15]Partec GmbH, Muenster 48161, Germany

[16]Department of State Standards in the Field of Physical-Chemical Measurements, VNIIM (D.I. Mendeleev Institute for Metrology), Moskovsky Pr., 19, 190005, St-Petersburg Russia and Biomedical Technologies, Institute of Cytology, Russian Academy of Science, 194064 St-Petersburg, Russia

Received 20 June 2014; Revised 16 December 2014; Accepted 30 December 2014

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

*Correspondence to: Lili Wang, National Institute of Standards and Technology, 100 Bureau Drive, Stop 8312, Gaithersburg, MD 20899-8312, USA.E-mail: lili.wang@nist.gov

Published online 5 February 2015 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22634

(CD4+) T cell is the primary target for the Human Immunodeficiency Virus-1 (HIV-1) and the infection causes a characteristic decrease in the number of circulating CD4+ T cells. According to the current WHO guidelines a CD4+ cell count of <350 cells per microliter of blood is diagnosed as acquired immune deficiency syndrome and is an indicator for antiretroviral therapy (1,2). On the other hand, Poncelet et al. reported that the surface CD4 expression level still remained constant on T helper cells of HIV-infected individuals even though the number of CD4+ T cells decreases in the progression of HIV-1 viral infection (3). Thereafter, multiyear research has supported the view that constant CD4 expression level on T cells can be used as a biological calibrator for the quantification of other surface and intracellular proteins for clinical diagnostics and immunotherapies (4–7).

Quantitative multicolour flow cytometry, incorporating labelled antibodies and fluorescence detection method, plays a critical role for the diagnosis of hematologic malignancies and immune disorders (8–12). 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) (13–15). One way to accomplish this goal is to use a biological reference cell which is known to possess a fixed number of well characterized protein markers such as CD4. Assuming a linear fluorescence intensity scale, a comparison of the fluorescence intensities from the reference cell and the test lymphocytes provides an estimate of ABC for the test lymphocytes. Currently there are no internationally recognized or validated biological reference standards though the development of biological reference materials (16) and practice guidelines for validating cell-based fluorescence assays (17,18) are emerging. This work suggests a candidate for a cell reference material.

A surface-labelled lyophilized cell preparation (sLL) has recently been developed by the National Institute of Biological Standards and Control (NIBSC), using human peripheral blood mononuclear cells (PBMC) pre-labelled with a FITC conjugated anti-CD4 monoclonal antibody (anti-CD4 FITC). The sLL is intended to be used as a reference counting standard for HIV/AIDS monitoring, and as a reference biological control with a known CD4 expression level for the FITC channel of flow cytometers. The aim of the present study, designated CCQM-P102, was to assess the ability of diverse investigators to quantify the CD4+ cell concentration and CD4 expression level of this reference cell material by flow cytometry. This report solely focuses on the measurements of CD4 expression level and associated measurement uncertainties.

For the measurement of CD4 expression level, participants were asked to construct a fluorescence intensity calibration curve for the FITC channel of a flow cytometer using commercially available calibration microspheres provided to all participants. The calibration microspheres have a very broad emission spectrum and provide a fluorescence intensity calibration for all of the fluorescence channels. The calibration microspheres consisted of five fluorescent populations each with a different loading of dye molecules. Each of the five populations was assigned a number of equivalent fluorescein fluorophores (*EFF*) which characterized the fluorescence intensity of that population. The *EFF* values were assigned at NIST using good laboratory practice documented in the guideline (18). The fluorescence intensity calibration curve was used to convert the measured geometric means of the fluorescence signals from sLL samples into *EFF* values characteristic of the level of CD4 expression. The participants were also asked to provide the raw data for centralized analysis by a single operator using a specific software tool to determine the effect of analysis on the variability of the results. We envisioned that a reasonably small uncertainty of the averaged *EFF* value from all participants would suggest that sLL may indeed be a viable biological standard. The positive result reported in this work motivates the next step which is a multiuser comparison of the *EFF* values from sLL reference cells and the *EFF* values from test lymphocytes. A similar but more exhaustive study in the future aims at multicolour flow cytometry measurements in units of ABC once the link between *EFF* and ABC is established for sLL. The ultimate goal of these studies is to integrate the quantification scheme into the clinical environment. For this reason it is important to demonstrate that all

Table 1. The *EFF* values and associated measurement uncertainties obtained for five fluorescent microsphere populations of ultra rainbow calibration microspheres

MICROSPHERE POPULATION[a]	*EFF* FOR FITC CHANNEL FC530	UNCERTAINTY IN *EFF*
Bead 5	5,607	1,200
Bead 4	42,070	9,190
Bead 3	117,400	26,500
Bead 2	251,100	58,000
Bead 1	543,500	126,000

[a]Note: the microsphere population numbering is consistent with the name shown in Figure 3 of the Supporting Information Document S2. The population number shown in Table 1 of the Supporting Information Document S1 was based on the gate defined in the FITC channel histogram of the microspheres in Figure 3 of the Supporting Information Document S1.

components of the quantification scheme work in a consistent manner, and that the associated measurement uncertainties are well understood.

Materials and Methods

Materials

The sLL material used in this study was described in detail in the companion manuscript of "quantification of cells with specific phenotypes I." Each participant was provided with six vials of sLL, six TruCount™ tubes of a single batch (Lot Number: 610431) from Becton–Dickinson (San Jose, CA), and a pack of the SPHERO™ Ultra Rainbow calibration microspheres that contained both blank and fluorescent microspheres (five different fluorescence intensity populations) from Spherotech (Lake Forest, IL).

Fluorescence Value Assignments of the Calibration Microspheres

The *EFF* values of the ultra rainbow calibration microspheres were assigned at NIST using fluorescein standard reference material (SRM) 1932 as the primary reference solution. The *EFF* values are given in Table 1 with a detailed assignment procedure provided in the Supporting Information Document S2. The uncertainties correspond to so-called combined uncertainties, taking into account various uncertainty contributions of the complex procedure needed for the assignment. The assignment of *EFF* values would be the responsibility of the microsphere manufacturer. A short summary of the process is given below for completeness sake.

Five serial dilutions of the stock fluorescein SRM were prepared gravimetrically using calibrated balances. The SRM solution is certified for fluorescein concentration. The fluorescence spectra of five dilutions of fluorescein SRM were measured, buffer subtracted, and relative spectral response correction was applied. The integrated fluorescence intensity associated with each dilution of the fluorescein SRM was determined by summing each of the spectra from 495 to 600 nm. The summation over the entire spectrum gave an unbiased representation of the fluorescence intensity. A calibration of the fluorimeter was obtained by plotting the logarithm of the concentrations (in mol L^{-1}) of the five dilutions of the fluorescein SRM on the y axis versus the logarithm of the corresponding integrated fluorescence intensities on the x axis. A linear least squares regression applied to the data, gave a good analytic representation of the relation between concentration of fluorescein and the corresponding fluorescence intensity. The fluorimeter calibration was performed in preparation for the analysis of the microsphere suspension fluorescence emission spectra taken under the same conditions as the spectra of the fluorescein solution. A spectrum from a suspension of undyed (blank) microspheres was used to subtract the background, and a correction for relative spectral response was performed. The fluorescence intensity associated with the microspheres in the FITC channel FC530 was found by summing each microsphere spectrum from 515 to 545 nm. Care was taken to identify the contribution from scattering of the exciting light at 488 nm. The equivalent concentration of reference fluorescein that gave the same fluorescence intensity as the microsphere suspension in the channel FC530 was defined as the value of concentration on the y axis of the intensity calibration line which corresponded to the fluorescence intensity of the microsphere suspension on the x axis.

The concentration of the microspheres was measured and the *EFF* was obtained by dividing the equivalent concentration of reference fluorescein by the microsphere concentration. That gave the number of equivalent fluorescein fluorophores, *EFF*, for a single microsphere.

The uncertainties in the values of *EFF* of the SPHERO™ Ultra Rainbow calibration beads were estimated by applying uncertainty propagation formulas to the entire sequence of measurements and are given in Table 1. The relative uncertainties in the *EFF* values are about 22% and reflect the long sequence of measurements needed to make the assignments. Efforts are underway to minimize these combined uncertainties.

Sample Preparation

The participants were requested to follow the standard protocol shown in Supporting Information Document S1. The lyophilised cells (sLL) were reconstituted by adding 1.0 mL of sterile distilled water to each vial and gently mixing, preferably on a roller, for 10–30 min before use. Three samples of the sLL diluted to one part in five using PBS (Dilution 1) were requested for the measurements of CD4 expression level. The total volumes of the test samples were 1 mL. The procedure specified that test sample tubes should be stored at 0–4 °C and acquired within 2 h after sample preparation.

Flow Cytometer QA/QC, Compensation, and Calibration

Flow cytometers used by participants were detailed in Supporting Information Table 1. Participants were required to perform routine instrument QA/QC of their choices. This QA/QC procedure ensures the optimum linear response range, the minimization of the contribution of the electronic noise, and cytometer settings adjusted for maximizing population resolution in each fluorescence detector. Once the flow cytometer performance was checked, participants were asked to ensure flow cytometers were properly compensated for

FITC and PE channels using their own choices of compensation methods. (The effect of compensation on the FITC channel was found to be minimal for this study which uses two labels, FITC and PE. See the Supporting Information Document S3). The sLL was labelled with FITC fluorophores for measuring the CD4 expression level. TruCount microspheres gave signals in both FITC and PE channels for determining the CD4+ cell count. Lastly, participants were requested to run ultra rainbow calibration microspheres for intensity calibration of flow cytometers and to ensure that the brightest fluorescent bead population was within the cytometer scale. Once the intensity calibration was performed for the FITC channel, the photomultiplier tube (PMT) voltage must not be changed during subsequent measurements. Immediately after calibration, the CD4+ cell counts and the geometric mean values of the fluorescence intensity of the CD4+ population were determined. Representative graphs of instrument compensation and FITC channel fluorescence calibration were provided in the standard procedure.

Sample Data Generation and Analysis

The procedure given in Supporting Information Document S1 specified that the forward scatter channel (FSC) or the side scatter channel (SSC) needed to be set properly for the sample data acquisition to detect TruCount microspheres (diameter ~3.8 μm) with sufficiently high signal to noise ratio. Representative gating strategy was provided for CD4+ cell counting in a dot plot of the side scatter channel (SSC) versus FITC channel. TruCount microspheres were identified in a dot plot of the FITC channel versus PE channel by a suitably chosen gate. CD4+ cells were identified by a gate in the dot plot of SSC versus FITC channels. The expression level of the CD4+ cells was measured by setting of a sub gate in the resulting FITC channel histogram and recording the geometric mean values of the gated CD4+ cells.

Using geometric mean values obtained for the calibration microspheres a fluorescence intensity plot was generated by plotting the microsphere geometric mean values versus *EFF* value provided in Table 1. The study procedure specified that the intensity calibration curve could be derived by using either a linear or logarithmic fit. For centralized analysis of participants' data, a non-linear fit to the logarithmic transformed data was performed using Eq. (1), where a and b are constant fitting parameters.

$$\log(EFF) = a + \log(\text{MeanChannel}) + \log\left(1 + b/10^{\log(\text{MeanChannel})}\right) \quad (1)$$

As discussed in reference (19), the slope of the corresponding linear relation is given by 10^a and the intercept by $10^a \cdot b$. The intensity calibration curve was used to calculate the *EFF* values of CD4 expression level. The *EFF* values of three individual samples were determined and reported.

Results

Analysis of *EFF* Results Submitted by Participants

The *EFF* values submitted showed inter-laboratory variation typically ranging from 15,000 to 25,000 with a minimum value of 6,354 and a maximum of 29,197. No outliers were observed for the mean values. Each participant determined the respective geometric mean channel value for Rainbow calibration microspheres and for CD4+ cells from the three sample vials. The geometric mean channel values of the calibration microspheres were used to generate a calibration curve for each participant using either a linear or logarithmic fit according to their preference. A representative calibration curve obtained for participant "B" with the logarithmic fit given in Eq. (1) is shown in Figure 1. The observed χ^2 associated with the fit in Figure 1 is $\chi^2_{obs} = 0.36$ and in the range $0.22 \leq \chi^2_{obs} \leq 9.35$ expected for a data set with 3 degrees of freedom, indicating that the Eq. (1) is a good representation of the data. The *EFF* values of CD4 expression were obtained by each participant using their calibration line. The results submitted by the study participants are summarized in Table 2 for three separate sample vials. The mean *EFF* values and uncertainties caused by pipetting and calibration of the data are included in Table 2. No outliers were identified when applying Grubbs' test at a level of confidence of 95%.

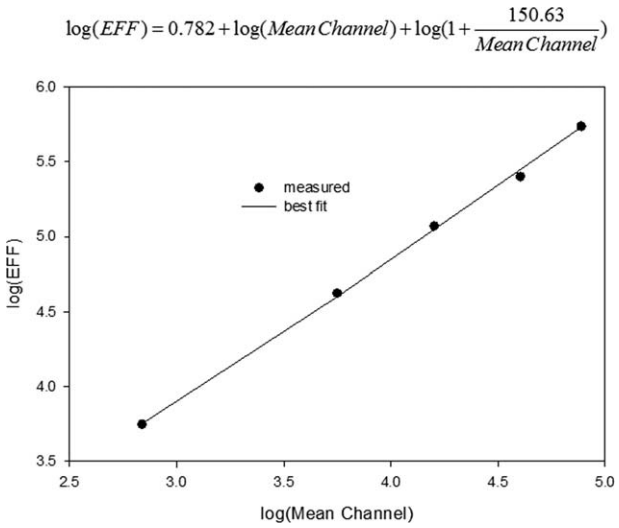

Figure 1. A calibration curve for the FITC channel of the flow cytometer for participant "B" is generated using five fluorescence intensity populations of the calibration microspheres and a non-linear fit to the logarithmic transformed data. The fitting equation with the best parameters is given above the plot. The χ^2 was calculated using the uncertainties in Table 1. The resulting value of χ^2 was 0.36, indicating an excellent fit for three degrees of freedom.

The mean *EFF* values derived from measurements of vials 1 to 3 submitted by the participants are shown in Figure 2a, as black dots. The (non-weighted) mean value is included in Figure 2a as a diamond. The data are also represented as box plot indicating the maximum value, 75% percentile, median, 25% percentile and minimum value.

Centralized Analysis and Comparison with Participants' Results

Centralized analysis was performed to identify additional inter-laboratory variation due to laboratory specific analysis as well as to identify any outliers. Some participants did not submit *EFF* results for all sample vials, and some participants did not calculate *EFF* values. However all participants

Table 2. The *EFF* values submitted by the study participants, and the mean *EFF* values and uncertainties derived from analysis of participants' reported *EFF* values

INSTITUTION	VIAL 1	VIAL 2	VIAL 3	MEAN	STANDARD DEVIATION	UNCERTAINTY	COMBINED UNCERTAINTY
A	20,608	20,904	20,525	20,730	155	120	149
B	23,320	24,080	22,560	23,320	760	135	459
C	np	18,920	18,359	18,640	397	132	310
D	18,202	np	np	18,202	0	182	182
F	11,407	11,693	10,804	11,301	454	65	270
H1	23,670	24,200	23,020	23,630	591	136	367
H2	19,290	20,090	19,580	19,653	405	113	260
I1	16,684	16,994	16,221	16,633	389	96	244
I2	16,066	16,375	15,757	16,066	309	93	201
J	15,212	15,212	14,432	14,952	450	86	274
K	26,289	27,588	26,127	26,668	801	154	487
L	6,450	6,275	6,338	6,354	89	37	63
M	22,673	22,644	22,193	22,470	248	130	193
N	21,872	21,507	21,514	21,631	209	125	174
O	29,879	28,701	29,012	29,197	610	169	391

Participating institution was labelled alphabetically. Institution "H" performed measurements on two different flow cytometers, and institution "I" requested a 2nd shipment of test samples and submitted two sets of data for two sets of test samples (All test samples were from the same sLL production lot manufactured at NIBSC, UK). The "np" means "not provided."

submitted raw data files in flow cytometry standard (FCS) format or list mode data (LMD) format. Based on these data, centralized analysis was performed by a single operator using FCS Express, version 3 from De Novo Software (Los Angeles, CA). The geometric mean channel values for Rainbow calibration microspheres were used to generate a calibration curve with the logarithmic fit given in Eq. (1). The calibration curve was used to obtain the *EFF* values of CD4+ cells from the measured geometric mean. The *EFF* values of CD4+ cells are summarized in Table 3 and depicted as a box plot in Figure 2b. Again, the (non-weighted) mean value is indicated by a diamond, the box represents the 75% percentile, median, and 25% percentile. The whiskers illustrate the maximum and minimum values. It follows from Figure 2 that centralized analysis revealed an outlier and a noticeable reduction in the variation of results between different laboratories.

There is one outlier identified by Grubbs' test at 95% confidence level for a particular participating institution "L" (Table 3). Two measurement issues were identified from the raw data files submitted by this participant (located in a high temperature region in the 2-month study period). An extremely high PMT voltage was applied to the FC530 during the data acquisition of the sLL such that part of lymphocyte and most of monocyte populations were off the scale. Second, both geometric mean values of CD4+ T cells and TruCount microspheres in the FITC channel histogram were fairly low even though geometric mean values obtained for the calibration microspheres were very similar to those shown in Figure 3 of the standard study procedure provided in Supporting Information Document S1. It's known that these hard-dyed calibration microspheres are much more stable at an elevated temperature than the lyophilized sLL, and the quantum efficiency of FITC is pH dependent (20). However, as the sLL were prepared in a common stock buffer and re-suspended only in water by the participants, the pH should be consistent in all study samples. Dilutions of sLL were carried out in PBS, which generally has a pH between 7.2 and 7.4 and therefore should have less effect on the FITC than measured by this participant (6). We therefore speculate the low geometric mean values of CD4+ cells obtained by this institution are likely due to a degradation issue of the sLL.

For comparison, the results of participants' and centralized analysis are listed in Table 4. Besides the non-weighted mean and its uncertainty we included the standard deviation, the number of measurements considered and the expansion factor k, which allows to calculate the expanded uncertainty (level of confidence 95%) from the standard uncertainty (level of confidence 68%). In addition, a χ^2 consistency test with an observed χ^2_{obs} value relating the sum of quadratic deviations from the mean value to the uncertainty of individual measurement (21) was performed. Although the centralized analysis gave improved results with respect to the standard deviation and the observed χ^2 value, the χ^2 test was found to be negative in both cases, since the requirements $5.6 \leq \chi^2_{obs} \leq 26.1$ (14 degrees of freedom) or $7.5 \leq \chi^2_{obs} \leq 30.2$ (17 degrees of freedom) were not met. The negative χ^2 test results suggest that not all influencing quantities were accounted for. The small standard deviations of typically 2% for individual participants' measurements (Table 2) were most likely caused by the combined effects of vial-to-vial variability and repeatability of the respective instruments. The large variations between the participants' results and hence the negative χ^2 test were not caused by the material provided, rather the deviations were due to systematic effects related to instrument specific differences, e.g., the variation in the spectral response of the filter–detector combination.

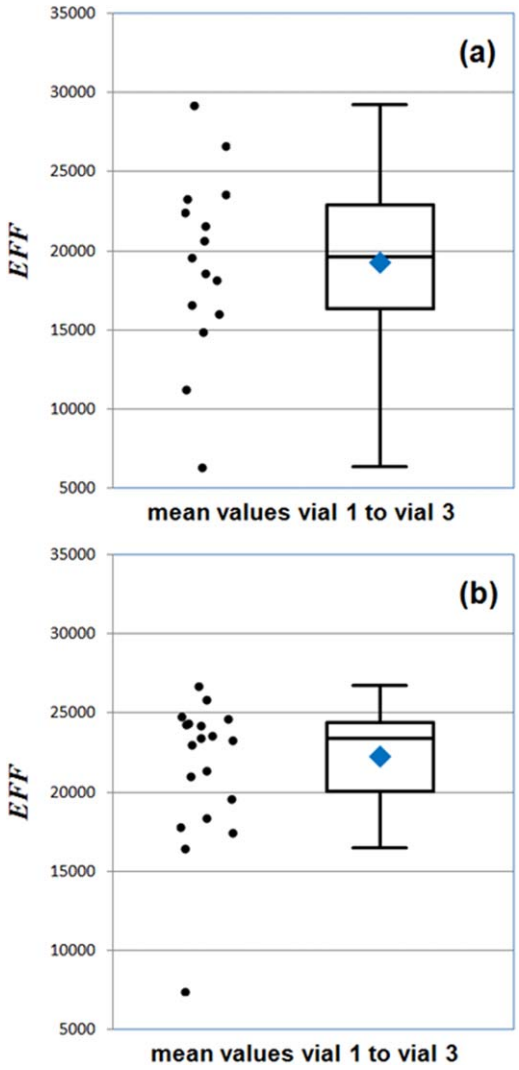

Figure 2. Comparison of the *EFF* values submitted by the study participants (**a**) and obtained by centralized analysis (**b**). Black dots represent the mean *EFF* values from participants. The mean values were derived from measurements of vial 1 to vial 3. The box plot indicates the respective maximum value, 75% percentile, median, 25% percentile and the minimum; the mean value of all participants is shown as a diamond. Using Grubbs test at 95% confidence level, there was no outlier detected from the *EFF* values submitted by participants in (a); however, one outlier was identified by centralized analysis shown in (b).

Discussion and Conclusion

We have performed an international trial to ascertain the capability for flow cytometric quantification of the antigen expression level of peripheral blood mononuclear cells in term of *EFF* values. To mimic fresh samples, which are not suited to an international collaborative study due to their inherent instability, stable sLL were sent to all participants. The mean *EFF* value derived from results submitted by the participating institutions was 19,296 (±1505) and the *EFF* value deduced from centralized analysis was 22,286 (±734) (Table 4). Because of the large variation of individual laboratory mean values no outlier was identified by Grubbs' Test (95% confidence level) in the *EFF* values submitted by participants. On the other hand, centralized analysis by a single operator resulted in a reduction of the standard deviation and rejection of one outlier value. The reduction in the *EFF* uncertainty by about a factor of two presumably resulted from a consistent gating strategy and uniform fitting algorithm. However, most likely there were some contribution to the large variability of the mean *EFF* values reported by individual institutions from analogue data collection (i.e., institution "F" and "O") and non-standard filters for FITC channel (wider than the standard FITC 515–545 nm filter, i.e., institution "G2-3" (505–545 nm filter) and "M" (510–560 nm filter) used in some of the flow cytometers (22,23). The effect of the centralized analysis resembles the results reported by Maecker et al. (24) and may articulate the usefulness of the automated flow cytometry data analysis techniques (25). In contrast, no reduction in uncertainty was noted when central analysis of CD4+ T cell counts was described in the companion manuscript quantification of Cells with Specific Phenotypes I.

The mean *EFF* value (22,286) obtained from the centralized analysis for the sLL were in good agreement with the antibody bound per cell value (21,000) reported for unstained lyophilized PBMC prepared by the same laboratory at NIBSC and stained with an unimolar anti-CD4 PE conjugate purchased from BD Biosciences (San Jose, CA) (16). Though the value of 22,286 is low relative to that measured using fresh PBMC, the sLL reference cell material with known CD4 expression level in terms of *EFF* value can still be very useful for obtaining comparable results with the use of FITC channel across different laboratories and instrument platforms. The good correspondence between the sLL and unimolar anti-CD4 PE stained unlabelled lyophilized PBMC (16) suggests that lyophilized unlabelled PBMC may also be a viable biological standard in multicolour cytometer measurements. In the latter case, the lyophilized PBMC has to be reconstituted and then stained with labels appropriate for the various fluorescence channels. It is likely in the future that both CD4 receptor density and the number of anti-CD4 antibody bound per CD4+ lymphocyte can be measured by using a mass spectrometry method we developed (26). This will ultimately enable the development of reference cell materials for quantitative multiparameter flow cytometric measurements. The use of reference cell materials in clinical laboratories will reduce measurement variations in the quantification of other surface and intracellular proteins for diagnostics and immunotherapies.

By labelling PBMC with anti-CD4 FITC antibody against domain 3 and 4 of the CD4 receptor, followed by stabilization and freeze-drying, a lyophilized cell standard was created with advantages of both lyophilized microspheres and cryopreserved PBMC or stabilized whole blood currently used for cross-laboratory standardization (27,28). This pre-labelled cell reference standard has the phenotypic characteristics of fresh PBMC, but without the need of constant monitoring to ensure their viability. The pre-labelled cell reference standard is sufficiently stable to permit shipment at ambient temperatures. This study demonstrated that the lyophilized and pre-

Table 3. *EFF* values derived by centralized analysis of the raw data files submitted

INSTITUTION	VIAL 1	VIAL 2	VIAL 3	MEAN	STANDARD DEVIATION	UNCERTAINTY	COMBINED UNCERTAINTY
A	21,030	21,330	20,940	21,100	204	122	170
B	23,320	24,080	22,560	23,320	760	135	459
C	*7,745*a	24,670	24,110	24,390	396	172	329
D	21,790	21,360	21,200	21,450	305	124	215
E	23,520	23,530	22,190	23,080	771	133	465
F	16,580	16,860	16,000	16,480	439	95	271
G1	24,230	23,350	25,270	24,283	961	140	572
G2	24,860	23,890	25,330	24,693	734	143	447
G3	25,880	25,280	26,490	25,883	605	149	380
H1	23,670	24,200	23,020	23,630	591	136	367
H2	19,290	20,090	19,580	19,653	405	113	260
I1	18,480	18,800	18,020	18,433	392	106	250
I2	17,860	18,170	17,550	17,860	310	103	207
J	17,760	17,760	16,980	17,500	450	101	279
K	24,470	25,690	24,320	24,827	751	143	457
L	7,595	7,418	7,481	*7,498*a	90	43	68
M	23,650	23,620	23,170	23,480	269	136	206
N	24,600	24,230	24,240	24,357	211	141	186
O	28,210	25,840	26,130	26,727	1,293	154	762

[a]Outlier identified using Grubbs test with a 95% confidence level.

Table 4. Mean value, uncertainty and standard deviation of the *EFF* values obtained from analysis of participants' submitted data and centralized data analysis

	NON-WEIGHTED MEAN	UNCERTAINTY OF MEAN VALUES	RELATIVE UNCERTAINTY OF MEAN VALUES	STANDARD DEVIATION	NUMBER OF MEASUREMENT VALUES	EXPANSION FACTOR *K*	χ^2_{obs}	CONSISTENCY TEST
Analysis of participants' data	19,296	1,505	7.8%	5,822	15	2.13	45,423	Negative
Centralized analysisa	22,286	734	3.3%	3,093	18	2.10	2,033	Negative

[a]One outlier was excluded from centralized analysis.

labelled PBMC cell reference material is suitable for clinical laboratories to calibrate and standardize CD4 enumeration for quality assurance of HIV/AIDS monitoring as well as quantitative expression analysis in the FITC channel. The inter-lab coefficient variance (CV) of 14% = $3.3\% \cdot \sqrt{18}$ (see Table 4) was obtained by centralized analysis of the CD4 expression measurements on identical samples of a single sLL production lot to which all participants only needed to add distilled water. The measurement variance is expected to be much larger when researchers and clinicians select their preferred reagents (antibody clones and fluorophore labels), staining procedures, lysis buffers and fixatives, instrument settings, gating strategies and methods of data analysis (28,29). It is therefore essential for the development of reference cell materials, e.g., sLL, and reference methods (30) to address at least some of these variables to achieve useful level of inter-lab result comparability. In our future studies, cell concentration and quantitative expression measurements will be extended to other important biomarkers, e.g., CD34+ for stem cells, and multiple fluorescence channels. Compensation, gating strategy, sample stability and photostability monitoring, and uniform fitting algorithm for calibration curve generation will be included in the study procedure to reduce measurement and analysis variability. In addition, the participant list will include clinical laboratories just like the study of CD34+ stem cell count currently under the planning and under the auspices of US Pharmacopeia.

Acknowledgments

The authors are indebted to Helen Parkes, Chair of CCQM/BAWG for her strong support of the study that lasted about 2 years and Dr. Sandrine Vessillier at NIBSC for her assistance during the manuscript review process. Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment identified are necessarily the best available for the purpose.

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