

Quantitative Flow Cytometry Measurements in Antibodies Bound per Cell Based on a CD4 Reference

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Multicolor flow cytometer assays with fluorescently labeled antibodies 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. Existing methods and cell reference materials for quantitative expression measurements have not yet produced results that are of wide clinical interest or are instrument-independent across all fluorescence channels. This unit details a procedure for quantifying surface and intracellular biomarkers by calibrating the output of a multicolor flow cytometer in units of antibody bound per cell (ABC). The procedure includes (1) quality control of the flow cytometer, (2) fluorescence intensity calibration using hard dyed microspheres assigned with fluorescence intensity values, (3) compensation for fluorescence spillover between adjacent fluorescence channels, and (4) application of a biological reference calibrator to establish an ABC scale. The unit also points out current efforts for quantifying biomarkers in a manner that is independent of instrument platforms and reagent differences. © 2016 by John Wiley & Sons, Inc.

Keywords: multicolor flow cytometry • fluorescence calibration • equivalent number of reference fluorophores (ERF) • CD4⁺ lymphocytes • antibody bound per cell (ABC)

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INTRODUCTION

Flow cytometry is a powerful immunological technique that can be used to assess complex biological processes at a single-cell level in heterogeneous populations. The complexity of the immune response necessitates measurements of as many cell receptors as practical for disease diagnostics and immunotherapies (de Vos van Steenwijk et al., 2010; Richards et al., 2012; Tembhare et al., 2013). An ultimate goal of quantitative flow cytometry is to measure the number of antigens or ligand-binding sites associated with a cell by



measuring the number of antibodies bound per cell (ABC). Determining the expression levels of cellular receptors/antigens requires quantitative measurements across all available fluorescence channels (FCs). Quantitative measurements have been implemented on the phycoerythrin (PE) channel using the QuantiBrite PE calibration approach (Iyer et al., 1998; Boonstra et al., 2006; Tembhare et al., 2013) and unimolar PE antigen conjugates. The availability of unimolar reagents is still a limiting factor. An international cell reference material of lyophilized peripheral blood mononuclear cells (PBMC) prelabeled with anti-CD4 fluorescein isothiocyanate (FITC) antibody has recently become available 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 (Stebbing et al., 2015; Wang et al., 2015). The PBMC reference material only enables quantitative expression analysis in the units of equivalent fluorescein fluorophore (EFF), because the link between EFF and ABC has not yet been established and is still under investigation. The purpose of this unit is to provide workable procedures that can lead to quantitative multicolor flow cytometry measurements in units of ABC. These quantitative measurements rely heavily on biological cell reference controls that are known to possess a fixed number of a well-characterized biomarker, for example, CD4 on healthy human T lymphocytes (Hoffman et al., 1993; Davis et al., 1998; Hultin et al., 1998; Wang et al., 2012; Degheidy et al., 2014). In addition, the availability of fluorescence solution standards will enable the calibration of flow cytometers and standardization of the fluorescence intensity scale of all fluorescence channels.

Bead sets are commercially available with manufacturer-assigned fluorescence intensity values based on procedures developed by each manufacturer. The intensity units used by the manufacturers may be traced to a solution of a fluorophore, be assigned an arbitrary value, or simply be expressed as a percentage of the intensity of the brightest bead in the set. Currently, none of the commercially available bead sets are traceable to an authoritative standards body such as the National Institute of Standards and Technology (NIST), and in the case of hard, dyed, non-spectrally matched beads there is no or limited information about how assigned values vary with excitation wavelength or emission spectral range.

Because of the urgent need for quantitative multicolor cytometry measurements, the NIST has developed a solution-based method to convert the fluorescence signal from fluorescent microspheres to the value of an equivalent number of reference fluorophores (ERF) using reference fluorophore standards such as fluorescein standard reference material (SRM) 1932 (NIST, 2007), Nile Red, Coumarin 30, and allophycocyanin (APC) (Wang et al., 2008). The ERF approach to fluorescence intensity units inherently includes information about the excitation wavelength and emission spectral range, as the assignments compare a defined excitation and emission spectral range for the reference fluorophore and beads (e.g., 488 nm excitation and 564-606 nm emission for beads intended to standardize the detector used for PE). This ERF-based calibration approach, including both spectrally (fluorescein and APC) and non-spectrally (Nile Red and Coumarin 30) matched fluorophore standards, was evaluated in a multi-site fluorescence standardization study co-sponsored by the International Society for Advancement of Cytometry (ISAC) and the NIST (Hoffman et al., 2012). The results of the joint study support the ERF-based calibration scheme and demonstrate limits of the utility of non-spectrally matched hard dyed microspheres for cytometer quality control (QC) and fluorescence calibration.

As discussed previously (Wang et al., 2008, 2011), quantitative cytometry measurements in the unit of ABC include two critical steps besides cytometer QC and compensation. In the first major step, the linearity of the FC is verified or determined (*UNIT 1.4*; Wood, 2009). Unless the instrument manufacturer provides an alternative means to determine

linearity, each of the cytometer FCs is standardized using a set of microspheres with assigned values such as ERF. The objective of this step is to establish a linear relation between the fluorescence signal in a given FC of multicolor flow cytometers and the value of ERF. The linearity calibration yields an estimate of the linearity of response and the dynamic range. Depending on the bead set used for linearity standardization, the detection efficiency, Q , can also be determined (*UNIT 1.20*; Hoffman and Wood, 2007). Q is defined as statistical photoelectrons (Spe) per signal unit, such as ERF. It is an important measure of instrument sensitivity and can be predictive of the success of the multicolor cytometry staining panel design (Perfetto et al., 2014). Moreover, the calibration and standardization in the unit of ERF enables the comparability of Q across various cytometer platforms and locations, regardless of microspheres used (i.e., from different manufacturers). This standardization facilitates studies and clinical trials across different laboratories and locations.

The second critical step is to use biological cell reference controls that possess a fixed number of CD4 receptors on a healthy individual's T lymphocytes to translate the linear ERF scale to the ABC scale. The biological reference control is incubated with antibodies labeled with one type of fluorophore for a particular FC, enabling the transformation of the ERF scale to the ABC scale for that FC (Wang et al., 2008, 2011). The biological reference marker CD4 receptor on human T helper cells can come from the whole blood of normal healthy individuals (which contains live red blood cells and nucleated cells) or from Cyto-Trol Control Cells, a commercially available lyophilized (dead) peripheral blood mononuclear cell (PBMC) preparation (Davis et al., 1998; Wang et al., 2012, 2014). The choice depends on user preference and the accessibility of whole-blood samples from normal healthy individuals. Cyto-Trol and PBMC preparations show different but reproducible CD4 expression levels in ABC using unimolar CD4 PE conjugates (SK3 clone, BD Biosciences). The expression levels are $\sim 45,000$ for fixed normal whole-blood samples and $\sim 40,000$ for Cyto-Trol cells (Wang et al., 2012). Due to antibody quality issues (including affinity binding dissociation constants, fluorophore labeling efficiencies, and fluorescence quantum efficiencies of the labeling fluorophores), it is essential at the beginning of a quantification study that anti-CD4 antibodies with different fluorophore labels be produced from the SK3 clone to establish the ABC scales (Davis et al., 1998; Wang et al., 2012). Once the ABC scales are put in place, users can then quantify test biomarkers relative to the CD4 reference marker using test antibodies (with the same fluorophore labels and high binding affinities and specificities) against the test biomarkers. Three published studies (Wang et al., 2006; Degheidy et al., 2014, 2015) have provided evidence supporting the assumption that different antibodies against different antigens with the same fluorophore label can have similar effective fluorescence per antibody values if the antibodies are produced by the same manufacturer.

The fluorescence spectra of most fluorophores used as labels cover a wide range of wavelengths. Consequently, a given label fluorophore may give a large fluorescence signal in the FC assigned to that fluorophore and smaller fluorescence signals in FCs assigned to other label fluorophores. Clearly the fluorescence signal in the FC not assigned to a given label fluorophore is a bias and should be corrected using a process called compensation (*UNIT 1.14*; Roederer, 2002). Compensation can be implemented using the biological cell reference controls individually stained with fluorescently labeled anti-CD4 antibodies. Software compensation on the data collected for each cell preparation including an unstained cell control should provide the necessary correction factors. Note that for flow cytometers equipped with FCs that have low detection efficiencies, cell reference controls labeled with anti-CD8 or anti-CD45 antibodies may be the choice for more accurate compensation correction. The CD8 and CD45 have higher expression level than CD4 on human lymphocytes and thus lead to higher fluorescence intensities.

STAINING CELLS FOR QUANTIFICATION OF CD20

Detailed steps are provided for staining whole blood samples and Cyto-Trol control cells. Unless otherwise indicated, all steps are performed at room temperature.

Materials

Freshly drawn whole blood from a normal healthy individual
1 × phosphate-buffered saline (PBS), pH 7.4 (Life Technologies, cat. no. 10010-023)

Fetal bovine serum (FBS; Sigma-Aldrich)

Fluorescently labeled monoclonal antibodies (BD Biosciences):

CD4 APC (SK3 clone, cat. no. 340443)
CD4 PerCP-Cy5.5 (SK3 clone, cat. no. 341654)
CD4 FITC (SK3 clone, cat. no. 340133)
CD4 PE-Cy7 (SK3 clone, cat. no. 348789)
CD4 V450 (SK3 clone, cat. no. 651850)
CD20 APC (L27 clone, cat. no. 340941)
CD20 PerCP-Cy5.5 (L27 clone, cat. no. 340955)
CD45 FITC (cat. no. 555482)
CD3 V450 (cat. no. 560365)
CD19 PE-Cy7 (cat. no. 560728)

Lysing solution, fixative-free (Life Technologies, cat. no. HYL-250)

1% (w/v) paraformaldehyde (Electron Microscopy Sciences) in 1 × PBS

Cyto-Trol control cell kit (Beckman Coulter), including reconstitution buffer

Disposable 12 × 75-mm polystyrene Falcon tubes (BD Biosciences)

Stain cells in fresh whole blood

- 1a. Wash heparinized normal healthy donor blood sample (6-8 ml) twice with ~40 ml of 1 × PBS, pH 7.4, containing 2% FBS in a 50-ml centrifuge tube. Centrifuge each time for 10 min at ~400 × g and remove plasma by aspiration. Replenish the original blood volume by adding 1 × PBS containing 2% FBS
- 2a. Aliquot 100 μl washed whole blood into individual 12 × 75-mm tubes.
- 3a. Add the desired mixtures of differently labeled antibodies to the tubes depending on the intended assays. Prepare an additional set of tubes, each with singly labeled anti-CD4 antibody (SK3 clone).

CAUTION: The tubes must be protected from light from this point forward.

Users can adopt the amount of antibody recommended by the manufacturer (e.g., for anti-CD4 from BD Biosciences, use 20 μl antibody per 100 μl whole blood) or can perform antibody titration curves to ensure that antibody staining is under saturation conditions. For titrations, start with the manufacturer's recommended amount of antibody, and do five 1.5- to 2-fold sequential dilutions plus one sample with one or two 1.5-fold increases over the manufacturer's recommendation. Choose the lowest concentration that gives nearly maximal fluorescence signal.

- 4a. Incubate 30 min at room temperature.
- 5a. Add 2 ml lysing solution and lyse cells for 10 min. Centrifuge 10 min at ~400 × g, then aspirate the supernatant.
- 6a. Wash once with 1 × PBS containing 2% FBS. Centrifuge 10 min at ~400 × g, then aspirate the supernatant.
- 7a. Add a small volume (<400 μl) of 1 × PBS containing 1% paraformaldehyde to fix cells and acquire samples immediately, or store tubes at 4°C and acquire within 2 hr.

Stain Cyto-Trol cells

- 1b. Reconstitute Cyto-Trol cells in a vial with 1 ml reconstitution buffer and mix gently for 10 to 30 min.
- 2b. Aliquot 100 μ l reconstituted Cyto-Trol cells into individual 12 \times 75-mm tubes.
- 3b. Add the desired mixtures of differently labeled anti-CD4 antibodies (SK3 clone) designated for each FC of a multicolor flow cytometer. Prepare an additional set of tubes, each with singly labeled anti-CD4 antibody (SK3 clone).

See notes following step 3a regarding antibody titration and protection from light.

- 4b. Incubate 30 min at room temperature.
- 5b. Wash cells with 2 ml of 1 \times PBS. Centrifuge 10 min at \sim 400 \times *g*, then aspirate the supernatant.
- 6b. Add a small volume (<400 μ l) of 1 \times PBS and acquire samples immediately, or store tubes at 4°C and acquire within 2 hr.

QUALITY CONTROL, CALIBRATION, AND INSTRUMENT COMPENSATION

Flow cytometer manufacturers generally provide QC microspheres and procedures for setting the instruments at optimal performance levels or at least qualifying instruments as operational. For instance, Cytometer Setup and Tracking (CS&T) Beads from BD Biosciences are used for QC of BD flow cytometers operated with Diva acquisition software. These microspheres consist of two different hard dyed fluorescent populations and an undyed blank population. The coefficient of variation (CV) of the brightest microsphere population is small enough that they can be used to assess laser alignment to the sample core stream of the flow cell. In addition, by adjusting the photomultiplier tube (PMT) voltage of each FC, the linear response range for each FC is estimated using the brightest microsphere population. The other fluorescent microsphere populations are designed to measure cytometer performance parameters such as photon detection efficiency (*Q*) and optical background (*B*). Note that unless the instrument has shown a linear response to fluorescence intensity, the measurement of *Q* can be inaccurate. After an initial QC of the flow cytometer, the performance of the cytometer can be tracked through the median fluorescence intensity (MFI) and associated CV of the brightest microspheres, as well as the PMT voltage change with respect to the initial voltage and *Q* and *B*. The hard dyed microspheres are stable over time at 4°C. These characteristics make the microspheres ideal for qualifying and tracking cytometer performance. Though general cytometry procedures for obtaining *Q* and *B* have been described by Chase and Hoffman (1998) and in *UNIT 1.20*, these two parameters are not evaluated as part of the daily instrument QC for some cytometer platforms. Users can derive *Q* and *B* for their own instruments following the procedures in *UNIT 1.20*.

The following protocol uses Ultra Rainbow microspheres (Spherotech) with assigned ERF values as an example for establishing a linear scale for fluorescence responses. The Ultra Rainbow microspheres consist of five fluorescent bead populations and one blank bead population. The linearity performance is carried out at an optimal and fixed PMT voltage of each FC.

NOTE: It is important to perform linearity calibration with hard dyed, non-spectrally matched beads prior to instrument compensation.

Calibrate flow cytometer fluorescence

1. Add two to three drops of both blank and fluorescent calibration microspheres to 0.5 ml PBS (\sim 5 \times 10⁴ to 5 \times 10⁵/ml).

BASIC PROTOCOL 2

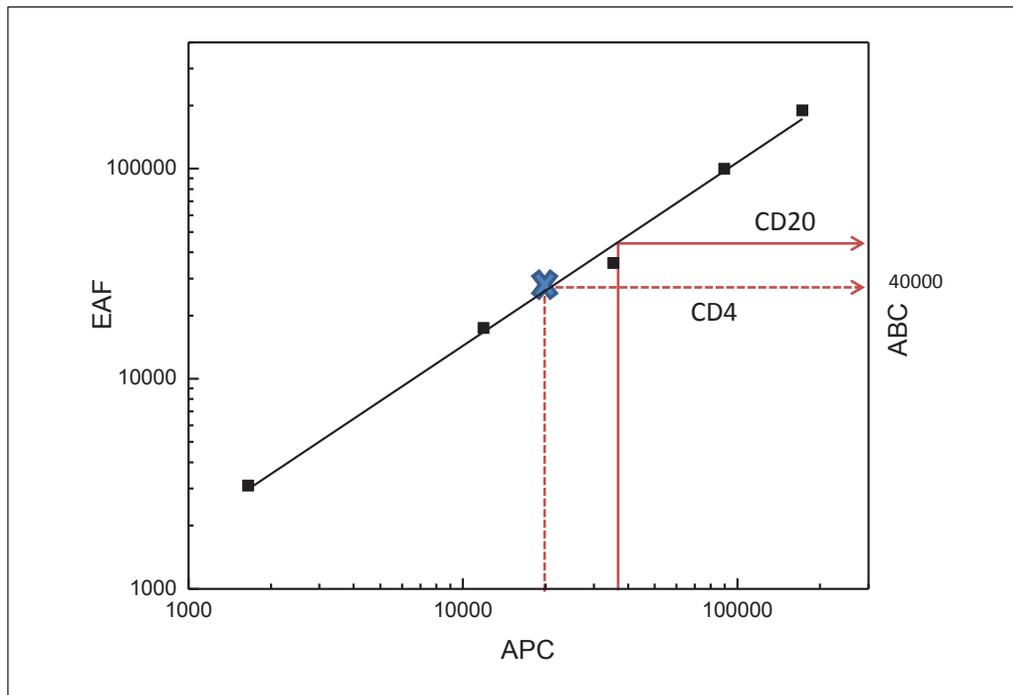


Figure 1.29.1 Example of a calibration curve for calibration microspheres plotted as MFI value (x axis) vs. number of equivalent APC fluorophores (EAF; left y axis) using the APC channel. The EAF values for five microsphere populations are provided in Table 2 of Wang et al. (2008) and in the Supplementary Materials (<http://www.currentprotocols.com/protocol/cy0129>). A linear fit is applied to the data set to produce an intensity calibration line for the APC channel of the multicolor flow cytometer. A vertical dashed line is drawn from the geometric MFI of CD4⁺ lymphocytes on the x axis to the calibration line. The blue X, where the dashed line crosses the calibration line, defines the EAF value corresponding to the number of labeled CD4 antibodies on the biological reference cells. This point sets the ABC scale (right y axis). The ABC value for fixed CD4⁺ lymphocytes from both whole blood and Cyto-Trol cells is ~40,000 (Davis et al., 1998; Wang et al., 2012, 2014; Degheidy et al., 2015). The solid line is a hypothetical connection between the MFI value from lymphocytes with labeled anti-CD20 antibodies and the corresponding ABC value.

2. Acquire 20,000 events within the most populated microsphere gate on the FSC vs. SSC dot plot. Make sure that the brightest fluorescent microsphere population lies within the quantifiable cytometer scale.
3. For analysis, use different gates in each FC histogram to obtain all MFI values for the calibration microspheres.
4. Construct a calibration curve of the calibration microspheres by plotting MFI value (x axis) vs. ERF value (y axis).

As an example, Figure 1.29.1 shows a linear calibration curve obtained for the APC channel using a linear fit to the data set for the APC channel of the multicolor flow cytometer. The ERF values for five microsphere populations are provided in Table 2 of Wang et al. (2008) and in the Supplementary Materials (<http://www.currentprotocols.com/protocol/cy0129>).

Perform instrument compensation

5. Carry out instrument compensation using unstained cells (whole blood or Cyto-Trol) or antibody capture beads plus cells or beads singly stained with CD4 FITC, CD4 PerCP-Cy5.5, CD4 PE-Cy7, CD4 APC or CD4 V450. Run unstained and stained samples following the cytometer manufacturer's compensation protocol and use the cytometer acquisition software to compute the compensation matrix.

QUANTIFICATION OF CD20 IN UNITS OF ABC

In this protocol, details are provided for converting an ERF scale to an ABC scale for reporting quantitative flow cytometry measurements. The procedure should be applicable for flow cytometers operated with the 405-, 488-, and 632-nm lasers commonly used in most flow cytometers, and appropriate dichroic mirrors and band pass filters to define the FCs.

The steps are followed by two examples for determination of CD20 expression in ABC units in whole blood from a healthy individual. The examples differ in the choice of CD4 reference: Example 1 uses T helper cells from the same healthy individual, whereas Example 2 uses T helper cells from Cyto-Trol cells.

Transforming the ERF scale to an ABC scale and obtaining quantitative measurements in ABC

1. Combine fluorescently labeled anti-CD4-stained whole blood samples or Cyto-Trol cells needed for the quantitation channels in a single sample tube.
2. If necessary, concentrate cells by centrifuging 10 min at $\sim 400 \times g$ to ensure that the number of CD4⁺ cells for each FC in the combined sample is about the same as the CD4⁺ cell number in the singly stained tube.
3. Run sample on the flow cytometer. Draw a singlet gate in the dot plot of FSC-A (area) vs. FSC-H (height) and a subsequent lymphocyte gate in a dot plot of CD45 vs. SSC-A. Acquire at least 20,000 events within the lymphocyte gate.
4. Draw a tight CD4⁺ gate on the fluorescence histogram in every FC used for quantification purposes and record the geometric MFI value of the CD4⁺ lymphocyte population for every FC.
5. As shown in Figure 1.29.1, draw a vertical dashed line from the geometric MFI of CD4⁺ lymphocytes in the figure containing the calibration curve of the calibration microspheres.

The point where the dashed line crosses the calibration line (blue X in Fig. 1.29.1) defines the ERF value corresponding to the number of labeled antibodies on the biological reference cells. This point sets the ABC scale on the right side of the y axis. The ABC value for fixed CD4⁺ lymphocytes from whole blood and from Cyto-Trol cells is $\sim 40,000$ (Davis et al., 1998; Wang et al., 2012, 2014; Degheidy et al., 2015).

6. Run unknown blood samples and obtain the geometric MFI values of unknown antigens of interest.
 - a. If the flow cytometer has been shown to have a proportional (strictly linear) relationship between MFI and fluorescence intensity within the range of values for the CD4 standard and unknowns, determine the ABC value of these unknowns as:

$$ABC_{\text{unknown}} = \frac{MFI_{\text{unknown}}}{MFI_{\text{CD4}}} \times ABC_{\text{CD4}}$$

Equation 1.29.1

- b. If a proportional response in the range used for CD4 calibration and the unknown is not demonstrated, use the actual response relationship.

When the flow cytometer uses a logarithmic amplifier, the most accurate results will be obtained using the log channel scale (e.g., 0 to 1024) rather than the scale that is converted to a decade scale (e.g., 1, 10, 100, 1000, 10,000; Hoffman, 2001). Conversion to the decade

scale assumes a nominal logarithmic response such as 256 channels per decade, which may not be accurate. Examples of alternative responses are:

For linear with an offset:

$$ABC_{\text{unknown}} = \frac{(\text{Slope} \times \text{MFI}_{\text{unknown}} + \text{intercept})}{(\text{Slope} \times \text{MFI}_{\text{CD4}} + \text{intercept})} \times ABC_{\text{CD4}}$$

Equation 1.29.2

Using log channel numbers for logarithmic amplifier data with proportional relationship between log channel number and log fluorescence intensity and with C channels per decade in the fit (Hoffman, 2001):

$$ABC_{\text{unknown}} = \frac{10^{\text{LogChannel}_{\text{unknown}}/C}}{10^{\text{LogChannel}_{\text{CD4}}/C}} \times ABC_{\text{CD4}}$$

Equation 1.29.3

Example 1: CD4 reference from same whole blood sample

The donor blood sample was split into two sample tubes. The test sample was labeled with CD45 FITC, CD19 PE-Cy7 to identify B cells, and CD20 APC to identify and quantify CD20 receptors on the B cells. First, a lymphocyte gate (R1; CD45⁺ and low SSC) was drawn in the plot of CD45 FITC vs. SSC-A (Fig. 1.29.2A). While gated on the lymphocytes, a dot plot of FSC-A vs. SSC-A then enabled the fine tuning of the lymphocyte gate (R2; Fig. 1.29.2B). Under the refined lymphocyte gate R2, the B cell gate was identified in a dot plot of CD45 FITC vs. CD19 PE-Cy7 (Fig. 1.29.2C). While gated on B cells, the CD20 histogram (Fig. 1.29.2D) shows the positive CD20⁺ gate used to obtain the geometric MFI value of CD20. The reference sample was stained with CD45 FITC, CD3 V450 to identify T helper cells, and CD4 APC to identify CD4 receptors on the T helper cells. As for the test sample, a lymphocyte gate was drawn in the plot of CD45 FITC vs. SSC-A and fine-tuned using in a dot plot of FSC-A vs. SSC-A. Under the fine-tuned lymphocyte gate R2, the T cell gate was identified in a dot plot of CD45 FITC vs. CD3 V450 (Fig. 1.29.2E). While gated on T cells, the CD4 histogram (Fig. 1.29.2F) shows the positive CD4⁺ gate used to obtain the geometric MFI value of CD4. With measured MFI values of CD20 (39,000) and CD4 (18,500), CD20 expression in ABC was calculated to be 84,300 using Equation 1.29.1.

Example 2: CD4 reference from Cyto-Trol cells

The same whole blood test sample was stained as described in Example 1. Cyto-Trol control cells were stained with CD45 FITC, CD3 V450 to identify T cells, and CD4 APC to identify CD4 receptors on the T helper cells. After staining and washing, the two cell samples were combined in a single tube and run on a calibrated flow cytometer. Two different gating strategies are shown in Figure 1.29.3. In strategy I, a large lymphocyte gate (CD45⁺ and low SSC) was drawn in the plot of CD45 FITC vs. SSC-A (Fig. 1.29.3A). While gated on the lymphocytes, CD4⁺ T cells and CD19⁺CD20⁺ B cells were identified in the dot plot of CD19 PE-Cy7 vs. CD20/CD4 APC (Fig. 1.29.3B). Alternatively, CD4⁺ T cells and CD20⁺ B cells can be identified in a dot plot of CD3 V450 vs. CD20/CD4 APC (Fig. 1.29.3C). The MFI values of CD20 and CD4 were obtained from the CD20/CD4 histogram (not shown) under the respective CD20⁺ B cell and CD4⁺ T cell gates. In strategy II, two individual lymphocyte gates (CD45⁺ and low SSC) were drawn as 'Cyt' for Cyto-Trol cells and 'Lymph' for the unknown whole blood sample in the plot of CD45 FITC vs. SSC-A (Fig. 1.29.3D). While gated on 'Cyt', T cells were identified in the dot plot of CD45 FITC vs. CD3

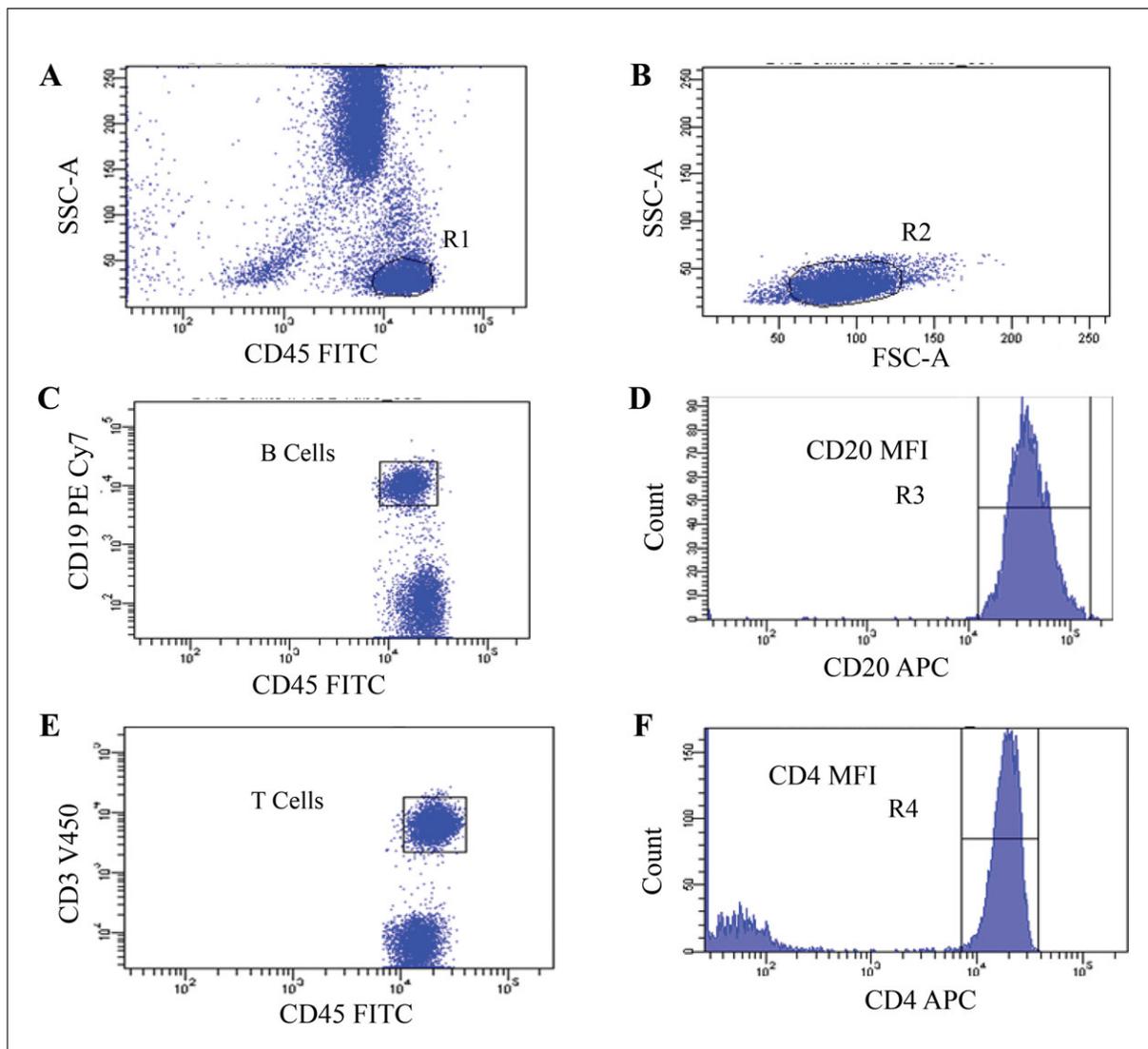


Figure 1.29.2 Quantification of CD20 expression level in ABC units based on known CD4 expression on T helper cells from healthy individual whole blood. The whole blood sample was split into two tubes. **(A-D)** The first tube was labeled with CD20 APC and antibodies that identify B cells (CD45 FITC, CD19 PE-Cy7), and was analyzed for unknown CD20. **(A)** A lymphocyte gate (CD45⁺ and low SSC) was drawn as R1 in CD45 FITC vs. SSC-A. **(B)** Gated on R1, a dot plot of FSC-A vs. SSC-A was used to draw a refined lymphocyte gate, R2. **(C)** Under the refined lymphocyte gate, a B cell gate was identified in a dot plot of CD45 FITC vs. CD19 PE-Cy7. **(D)** Finally, while gated on B cells, the CD20 histogram shows the positive CD20⁺ gate (R3) used to obtain the geometric MFI value of CD20. **(E-F)** The other tube was labeled with CD4 APC and antibodies that identify T cells (CD45 FITC, CD3 V450), and was defined as the CD4 reference. As for the unknown CD20 sample, a lymphocyte gate was drawn in CD45 FITC vs. SSC-A and a fine-tuned lymphocyte gate was then obtained in a dot plot of FSC-A vs. SSC-A (not shown). **(E)** Under the fine-tuned lymphocyte gate, the T cell gate was identified in a dot plot of CD45 FITC vs. CD3 V450. **(F)** While gated on T cells, the CD4 histogram shows the positive CD4⁺ gate (R4) used to obtain the geometric MFI value of CD4. With measured MFI values of CD20 and CD4, CD20 expression in ABC can be calculated using Equation 1.29.1.

V450 (Fig. 1.29.3E). Under the T cell gate, the CD4 histogram shows the positive CD4⁺ gate (Fig. 1.29.3F), which was used to obtain the MFI value of CD4. While gated on 'Lymph', B cells were identified in the dot plot of CD45 FITC vs. CD19 PE-Cy7 (Fig. 1.29.3G). Under the B cell gate, the CD20 histogram shows the positive CD20⁺ gate (Fig. 1.29.3H) used to obtain the MFI value of CD20. With measured MFI values of CD20 (38,100) and CD4 (17,700), CD20 expression in ABC was calculated to be 86,100 using Equation 1.29.1.

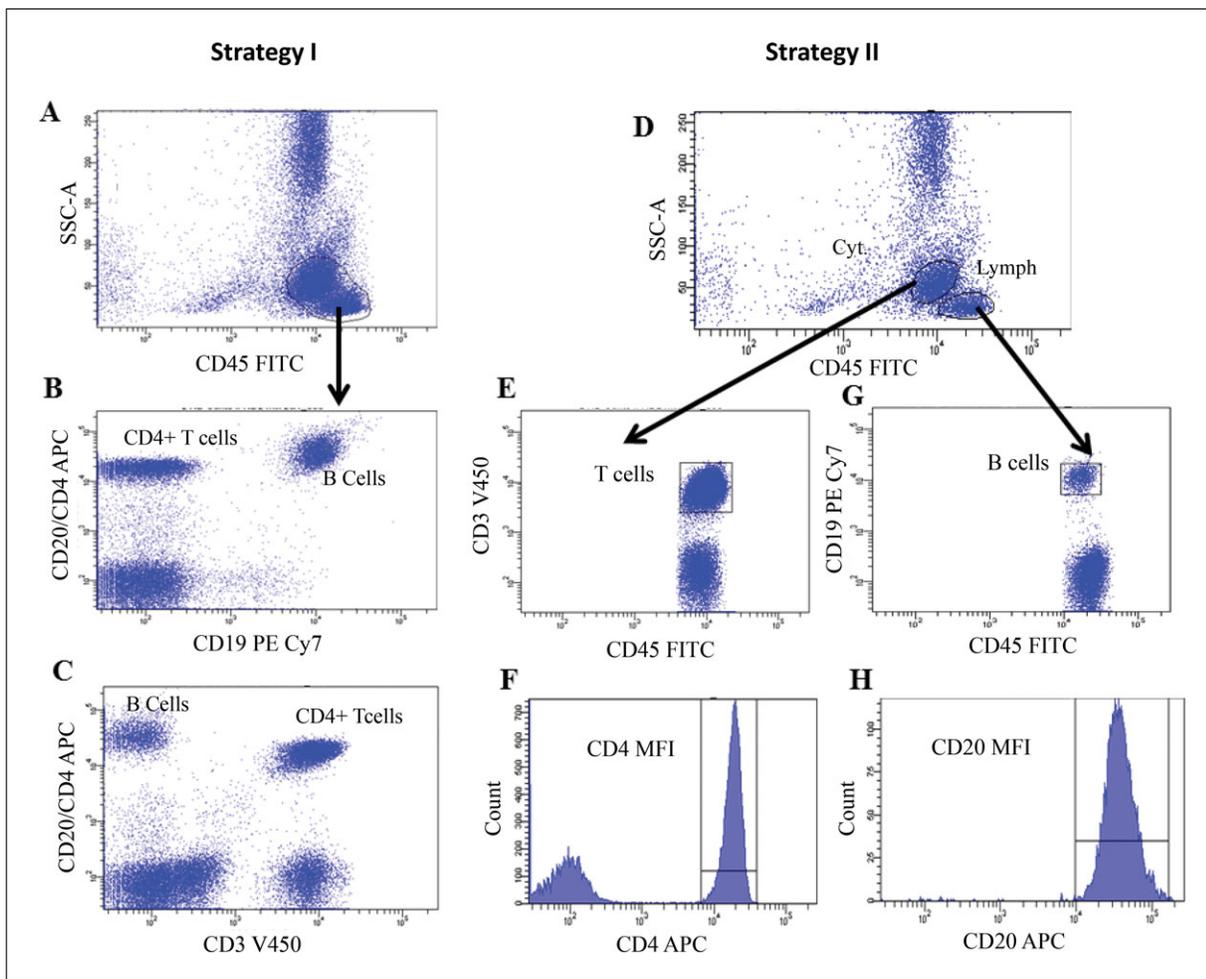


Figure 1.29.3 Quantification of CD20 expression level in ABC units based on known CD4 expression on T helper cells from Cyto-Trol control cells, both stained in APC. The whole blood sample was stained with CD45 FITC, CD19 PE-Cy7, and CD20 APC. In a separate tube, Cyto-Trol cells were stained with CD45 FITC, CD3 V450, and CD4 APC. After staining and washing, the samples were combined in a single tube and run on a calibrated flow cytometer. Two different gating strategies are shown. Strategy I: (A) A large lymphocyte gate (CD45⁺ and low SSC) was drawn in CD45 FITC vs. SSC-A. (B) Gated on lymphocytes, CD4⁺ T cells and CD19⁺CD20⁺ B cells were identified in a dot plot of CD19 PE-Cy7 vs. CD20/CD4 APC. (C) Alternatively, CD4⁺ T cells and CD20⁺ B cells can also be identified in a dot plot of CD3 V450 vs. CD20/CD4 APC. The MFI values of CD20 and CD4 can then be obtained from CD20/CD4 histogram under the respective CD20⁺ B cell and CD4⁺ T cell gates. Strategy II: (D) Two individual lymphocyte gates (CD45⁺ and low SSC) were drawn as 'Cyt' for Cyto-Trol cells and 'Lymph' for the unknown whole blood sample in CD45 FITC vs. SSC-A. (E, F) Gated on 'Cyt', T cells were identified in a dot plot of CD45 FITC vs. CD3 V450 (E). Under the T cell gate, the CD4 histogram (F) shows the positive CD4⁺ gate used to obtain the MFI value of CD4. (G, H) Gated on 'Lymph', B cells were identified in a dot plot of CD45 FITC vs. CD19 PE-Cy7 (G). Under the B cell gate, the CD20 histogram (H) shows the positive CD20⁺ gate used to obtain the MFI value of CD20. With measured MFI values of CD20 and CD4, CD20 expression in ABC can be calculated using Equation 1.29.1.

COMMENTARY

Background Information

Multicolor flow cytometer assays are routinely used in clinical laboratories to measure the cell numbers of specific phenotypes and to estimate the specific receptor/antigen expression levels on the surface or interior of cells taken from blood, lymph nodes, and bone marrow. The cell numbers and specific receptors/antigens serve as biomarkers

for pathological conditions at various stages of a disease. Moreover, flow cytometry measurements critically support many clinical, pharmacologic, and calibration and measurement capability claims used for drug, device, and biologics product development and approval. Hence, reference materials for cytometer standardization and quantitative measurements are in great demand for empowering

multicolor flow cytometry in medicine and healthcare.

A reference fluorophore solution standard kit, designated SRM 1934, is currently in production at the NIST. This standard kit includes fluorescein SRM 1932, Nile Red, Coumarin 30, and APC. It will allow fluorescence value assignments of fluorescently labeled microspheres in the unit of ERF. Standardization in the unit of ERF enables the comparability of the fluorescence intensity/channel scale of different flow cytometers and quality of flow cytometer performance (e.g., *Q* and *B*), enormously facilitating experimental/panel design across laboratories at different locations in clinical trials.

As illustrated here, biological cell reference controls that possess a fixed number of CD4 receptors on human T lymphocytes are needed for transformation of a linear ERF scale to an ABC scale. The use of cell reference controls enables the quantitative measure of biomarker expression levels in units of ABC independent of the flow cytometer used. The expression level of the CD4 reference marker on Cyto-Trol control cells has been measured to be ~40,000 using the well-characterized anti-CD4 antibody clone SK3 (Estess et al., 1990; Davis et al., 1998) by quantitative flow cytometry and mass cytometry (Wang et al., 2012). Moreover, the CD4 receptor density on CD4⁺ lymphocytes from Cyto-Trol cells measured by quantitative mass spectrometry in the presence of isotope-labeled, full-length recombinant CD4 receptor protein as the internal quantification standard is in a good agreement with the value estimated by quantitative flow cytometry (Wang et al., 2014).

For the ABC scale transformation in every FC of flow cytometers equipped with 405-, 488-, and 633-nm laser excitations, it is essential that the affinity binding dissociation constant (K_d) of the SK3 anti-CD4 antibody clone is highly specific and not affected by labeling of different fluorophores. The fluorophore labeling effect on the K_d for the SK3 clone of anti-CD4 has been investigated by performing titration curves using thawed PBMCs according to:

$$\frac{1}{F} = \frac{1}{F_{\max}} + \frac{K_d}{F_{\max}} \times \frac{1}{C}$$

Equation 1.29.4

where *F* is the MFI of CD4⁺ lymphocytes, F_{\max} is the MFI under the saturation staining condition, and *C* is the concentration of

Table 1.29.1 Affinity binding dissociation constants and associated standard deviations for the SK3 clone of anti-CD4 monoclonal antibodies labeled with different fluorophores^{a,b}

Antibody (cat. no.)	K_d (nM)	SD
CD4 FITC (340133)	0.129	0.005
CD4 PE (347327)	0.142	0.019
CD4 PerCP-Cy5.5 (341654)	0.923	0.092
CD4 PE-Cy7 (348789)	0.102	0.011
CD4 APC (340443)	0.098	0.010
CD4 APC-Cy7 (341095)	0.170	0.018
CD4 V450 (651850)	0.123	0.017
CD4 V500-C (647456)	0.305	0.021

^aValues obtained by performing titration curves on thawed PBMCs and analyzed by Scatchard plot on the basis of Equation 1.29.4.

^bAll fluorophores from BD Biosciences.

labeled anti-CD4 antibody in the staining reaction (Voet and Voet, 1990). After a linear fitting of the titration points in a plot of $1/F$ vs. $1/C$, K_d is calculated as the ratio of the slope and intercept of the fitted line. The K_d values for eight fluorescently labeled, off-the-shelf anti-CD4 monoclonal antibodies (all SK3 clone) are shown in Table 1.29.1. All of the binding constants are in the sub-nanomolar range. It is therefore expected that fluorophore labeling does not significantly alter binding of the antibodies to CD4 receptors on T lymphocytes.

Off-the-shelf anti-CD4 antibodies (Table 1.29.1) are cost effective and widely accessible, and enable biomarker quantification in all fluorescence channels for biological applications. It is worth stressing that unconjugated antibodies are likely present in off-the-shelf antibody reagents and will bind more strongly to CD4 receptors on the surface of helper T cells, thereby out-competing antibody labeled with large fluorescent proteins (e.g., PE) and resulting in lower MFI values. It was found that the geometric MFI obtained for CD4 expression from normal healthy individuals and Cyto-Trol cells (done in triplicate) were very similar using off-the-shelf anti-CD4 PE (SK3 clone) and a linearity-calibrated flow cytometer (35,500 [CV, 7%] for fixed normal whole blood samples; 35,900 [CV, 2.0%] for Cyto-Trol cells) (unpub. observ.). A slightly higher ABC value of 45,000 was reported for fixed whole blood of healthy individuals compared to 40,000 for Cyto-Trol cells (Davis et al., 1998) using

the unimolar CD4 PE conjugate of the same antibody clone. The lower fluorescence intensity value from whole blood samples is probably due to the lower reagent purity of the off-the-shelf CD4 PE conjugate and/or antibody binding hindrance of the whole blood samples (Wang et al., 2014). Hence, due to the use of the off-the-shelf anti-CD4 antibodies, the ABC values for fixed CD4⁺ lymphocytes from whole blood and from Cyto-Trol cells are set to ~40,000 (Fig. 1.29.1), consistent with the results of Cyto-Trol cells from flow and mass cytometry, and quantitative mass spectrometry.

The ratio method for obtaining the expression level of the unknown (target biomarker) in ABC (Eqn. 1.29.1) assumes that the labeled antibody against the unknown and labeled anti-CD4 antibody have similar effective fluorescence per antibody values (so called effective *F/P*). Several studies support the assumption that different antibodies against different antigens with the same fluorophore label can have similar effective *F/P* values if these antibodies are produced by the same manufacturer (Wang et al., 2006; Degheidy et al., 2014, 2015). However, this assumption has not been verified with results of unknown antibodies with the same fluorophore label produced by different manufacturers. It would be valuable to determine whether monoclonal antibodies produced by different manufacturers (often from different clones) with the same fluorophore label and similar affinity binding constants against the same test antigen produce similar results. On the other hand, it is feasible that single-photon-based detection techniques (Eisaman et al., 2011) could be applied to measure individual fluorescently labeled antibodies and cells stained with the same labeled antibody for the determination of its ABC value, and evaluate the purity of labeled antibody reagents. Moreover, *UNIT 1.30* (Kantor et al., 2016) presents an alternative method for evaluating the brightness of antibody-dye reagents and estimating antibodies bound per cell. Their approach provides comparisons of the brightness of different antibody reagents that use the same dye, and also allows brightness comparisons among reagents utilizing different dyes when conducted on an instrument for which the statistical photoelectron (Spe) scales are known.

Critical Parameters and Troubleshooting

QC is extremely important for successful quantitative flow cytometry measurements.

Because disease biomarkers are often expressed at low levels, users must carefully choose the fluorophores/FCs and antibody reagents to be used for quantification. A general principle would be to use FCs with the highest *Q* and lowest *B* possible for biomarkers with low expression levels. Moreover, fluorescence calibration of flow cytometers using calibration microspheres most importantly ensures the linearity of fluorescence responses of the FCs to be used for quantification, which is essential for the determination of unknown expressions on the basis of Eqns. 1.29.1-1.29.2. The multiple microsphere population gates in the FC histograms can be used to periodically track the stability of cytometer performance with the PMT voltages of all FCs kept the same as the original voltage setting after flow cytometers pass daily QC. While this window of analysis shows the identical instrument performance under the same operational parameters (PMT voltages and filter sets), users may transfer CD4 reference biomarker expression in ABC to the calibration microspheres in their first experiment and use the calibration microspheres with established ABC values for quantitation of unknowns during subsequent experiments. Large deviations in the window of analysis of any FC would require attention for troubleshooting and/or instrument service.

Proper cytometer compensation is critical for quantification of target biomarker expressions, especially when CD4 reference marker and target analyte reside in different sample tubes. If the flow cytometer does not display CD4 in the middle-to-upper range of the channel scale, which allows the most accurate compensation setting, users could consider using cell reference controls labeled with brighter anti-CD8 or anti-CD45 antibodies for more accurate compensation correction.

It is well known that quantitative flow cytometry measurements of biomarker expression levels depend on many variables, such as sample type staining protocol, fixation conditions, antibody clones, and fluorophore and conjugation chemistries. It is crucial to choose antibody reagents with high affinity binding dissociation constants against antigens of interest and low probabilities of nonspecific binding. These can be assured by performing titration curves and assessing nonspecific staining with appropriate isotype controls, or by using fluorescence minus one (FMO) controls to test each individual component of a staining panel (*UNIT 1.14*). Sample staining protocols ought to be optimized for best and reproducible results.

Anticipated Results

The production of fluorescently labeled cell reference materials with known ABC values of a reference biomarker will require large quantities, accurate biomarker expression levels measured by two different techniques, resemblance to clinical samples, long-term stability, and low-cost storage conditions. This protocol outlines a general procedure that will allow users to measure the expression levels of unknowns in units of ABC, independent of the flow cytometer used. It is envisioned (with supporting evidence found in Degheidy et al., 2014, 2015) that the use of the well-studied CD4 reference biomarker will enable users to quantify target biomarker expression in ABC with significantly lower CVs than those associated with the unknown expressions in MFI that are mostly reported at present and are dependent on the instruments used.

Time Considerations

Sample preparation including pipetting, staining, red blood cell lysis, and cell washing, consumes ~2 to 4 hr and is highly dependent on the number of samples/tubes. The flow cytometer QC, performed with QC beads provided by the instrument manufacturer and analyzed by the instrument acquisition software, should not take more than 10 to 15 min. It may take 1 to 2 hr to perform flow cytometer QC by running microspheres of your own choice and performing calculations for Q and B . If the configuration of the flow cytometer is not modified, Q and B should not change from day to day. Flow cytometer compensation and calibration in the units of ERF takes 15 to 20 min. Again, the total time for sample acquisition depends on the number of samples, cell density, and number of events collected. Data analysis should take ~1 hr, assuming that the gate strategy has already been developed.

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