Flow cytometric CD4 enumeration of four different HIV-Infected blood samples at the cost of one monoclonal antibody reagent

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Summary

Background: The frequency and absolute number of CD4⁺ T-lymphocytes continue to be one of the major clinical markers for management of HIV/AIDS. The present standard dual-platform (DP) three-color and two-color PanLeucogating flow cytometric (FCM) methods for most developing countries are either expensive if manufacturers’ monoclonal antibody reagents are used or limited due to an insufficient supply of generic reagents. Clearly, more affordable FCM methods are needed.

Objective: To develop a novel DP FCM method using biotin-streptavidin-fluorochrome labeling in combination with the two standard DP methods for 4 different white blood cells (WBC) using only one monoclonal antibody reagent.

Methods: The percentage of CD4⁺ T-lymphocytes in 116 HIV-infected blood samples was determined using our new method. Results were compared with the two standard methods. Correlation and agreement of the pair method were determined using linear regression, Bland-Altman and percent similarity analysis.

Results: Our study showed that percentage of CD4⁺ T-lymphocyte values obtained from the new method correlated highly with the standard three-color and the two-color methods (r² = 0.95 (n =52) and 0.97 (n =64)). The mean bias and percent similarity for the new method compared with the two standard methods were -0.53% (limit of agreement {LOA}:-5.22% to +4.16% with percent similarity of 99.28; and -0.22% with LOA of -3.42% to +2.98%, the percent similarity of 98.15, respectively.

Conclusions: Our FCM method using biotin to label 4 different WBC samples followed by streptavidin staining is reliable for determination of CD4⁺ T-lymphocytes. Such an approach will significantly reduce the cost for monitoring HIV-infected patients in resource-limited settings. (Asian Pac J Allergy Immunol 2011;29:190-9)

Key words: acquired immunodeficiency syndrome; biotin; CD4 T-Lymphocytes; flow cytometry; HIV

Introduction

The recent introduction of inexpensive and generic anti-retroviral therapy (ART) has been an important step in the fight against HIV/AIDS in resource-limited settings.¹ However, adequate management of persons infected with HIV in these countries is possible only if ART is accompanied by accessible and affordable laboratory monitoring in the form of CD4⁺ T-lymphocyte enumeration. Although plasma viral load is considered to be more informative for guiding decisions regarding the timing of implementation of ART, it is expensive and/or not available in resource-constrained countries.

CD4⁺ T-lymphocytes are the primary target of HIV-1 and are gradually depleted during the course of the disease.²³ It is generally accepted that ART should be started in HIV-infected persons before the peripheral CD4⁺ T-lymphocyte level falls below 200 cells/µl.²⁴ Therefore, CD4⁺ T-lymphocyte levels are frequently monitored to assess immune competence and disease progression in HIV-infected persons.

Flow cytometry (FCM) is the most accepted standard for enumeration of CD4⁺ T-lymphocytes because of its accuracy, precision and reproducibility.⁷¹³ Recently, simpler strategies employing fewer fluorescence-conjugated monoclonal antibodies (i.e., PanLeucogating and
primary CD4 gating) have been described. In the past decade, supporters of the PanLeucogating strategy have advocated the use of total leucocyte counts and enumerating the frequency of CD4⁺ T-lymphocytes among CD45 leucocytes. During the past few years, several groups including our own have demonstrated that an excellent agreement exists between this PanLeucogating method and the standard methods.

Standard FCM CD4 testing can be performed using either a dual-platform (DP) or single-platform (SP) method. The DP approach uses two instruments in which an absolute CD4⁺ T-lymphocyte count is derived by multiplying the FCM percentage of CD4⁺ T-lymphocytes by the absolute lymphocyte count from a hemato logical cell analyzer. By contrast, the SP approach produces absolute CD4⁺ T-lymphocyte counts without the need for a hematological cell analyzer. Unfortunately, the use of bead-based FCM for the SP approach is still expensive (US$ 20-25) when compared to the DP FCM system using a hematological analyzer (US$ 15-18) as it requires an additional cost of US$ 4-5 for the reference microbeads either in the form of lyophilized pellets or liquid suspension. Consequently, FCMs using the DP approach are still the preferred choice in many resource-limited countries including Thailand and India despite their many drawbacks. This method is inconvenient, inaccurate and there is wide intra-laboratory variation, as reported by the United Kingdom National External Quality Assessment Scheme (UKNEQAS) for immunophenotyping systems.

In an effort to identify and implementing a relatively more economical, accurate and reproducible assay, not only for enumerating CD4⁺ T-lymphocyte counts but also a number of related assays, has been devised by our laboratory. Recently, a marked reduction in costs of reagents by dilution technique has been achieved in FCM based determination of CD4⁺ T-lymphocytes. This approach is promising and provides an affordable alternative method for resource-limited settings like Thailand. In this study we described another affordable approach utilizing 4 different blood samples which are simultaneously stained with one standard volume of staining reagents recommended by the manufacturer in a single tube. This is achieved by labeling 3 different blood samples with different concentration of biotin which are then mixed with one unlabeled blood sample. The mixture of 4 different blood samples is subsequently stained with a set of three-color or two-color monoclonal antibody reagents. We used this novel FCM method to enumerate the percentage of CD4⁺ T-lymphocytes and compared the results with the values obtained by the standard three-color and the two-color PanLeucogating FCM methods.

**Methods**

**Patients and blood samples**

Peripheral blood samples from 116 patients at various stages of HIV-1 infection were evaluated in this study. Two mL of venous blood from each patient was collected by venipuncture into K₂EDTA-containing tubes and processed for immunophenotyping within 8 hours. All HIV-infected blood samples were leftover from routine clinical samples that were unlinked from identifiers and tested at the Department of Immunology, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand. This study was approved by the Ethics Committee of the Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

**Reagents and monoclonal antibodies**

A water soluble, sulfo-succinimidyl-6-(biotinamido) hexanoate (NHS long-chain biotin) or succinimidyl biotin (NHS biotin) was purchased from Pierce (Rockford, IL). Various working concentrations of biotin (0.001, 0.01, 0.1 mM) were prepared freshly in phosphate buffered saline (PBS: Sigma, St Louis, MO). Fluorescein isothiocyanate (FITC) and allophycocyanin (APC)-conjugated streptavidin were obtained from Becton Dickinson (BD)-Phar mingen (San Diego, CA) and eBioscience, San Diego, CA. Anti-human monoclonal antibody to CD45 conjugated with peridinin chlorophyll protein (PerCP), anti-human CD3-conjugated with FITC and anti-human CD4-conjugated phycoerythrin (PE) were commercially obtained from Becton Dickinson Biosciences (BDB: San Jose, CA) and used separately or in a combination of three differently coloured monoclonal antibodies (TriTEST™ reagent (BDB)). All monoclonal antibody reagents were utilized at the concentration recommended by the manufacturer.

**Biotinylation of blood samples**

Fifty μl of each whole blood sample were dispensed into 1 mL of 1x FACSLysing™ solution (BDB) and incubated for 10 min at room temperature. The lysed red blood cells were removed by centrifugation. The remaining white
blood cell (WBC) pellet was washed once with PBS and incubated with 50 μl of biotin at the various concentrations mentioned above for 10 min. A non-biotinylated WBC pellet was also obtained by adding 50 μl of PBS. After 30 min of incubation at room temperature, the cell pellets were washed with PBS and incubated with a pre-determined optimal concentration of FITC- or APC-conjugated streptavidin for another 30 min at room temperature. Following washing and centrifugation, each cell pellet was re-suspended in 20 μl of PBS. Twenty μl of each of the WBC pellets labeled with biotin at 0.001, 0.01 and 0.1 mm were mixed with 20 μl of the non-biotinylated WBC pellet. The mixture of these 4 different WBC samples was then utilized for subsequent immunophenotypic staining.

**Standard three-Color immunophenotyping method**

For the standard three-color FCM method, 10 μl of each individual monoclonal antibody against CD3, CD4 and CD45 conjugated with FITC, PE and PerCP, respectively, or 20 μl of TriTEST reagents and 50 μl of EDTA-antiocoagulated whole blood were added to a 12 x 75 mm Falcon polystyrene tube. The mixture was gently mixed and incubated for 20 min. at room temperature in the dark before adding 450 μl of FACSLysing solution. After 15 min of incubation, the lyse-no-wash stained samples were mixed and analyzed by FCM. For FCM analysis, data from each three-color monoclonal antibody, or TriTEST reagents-stained blood sample were acquired and analyzed with MultiSET™ software (BDB) on the FACS Calibur™ FCM (BDB).

**The PanLeucogating immunophenotyping method**

The PanLeucogating immunophenotyping staining of peripheral blood samples was performed by adding a pre-determined optimal 10 μl of monoclonal antibody to CD45 and CD4 conjugated with PerCP and PE to 50 μl of whole blood in polystyrene tubes. All tubes were gently mixed and incubated for 20 min. at room temperature in the dark before the addition of 450 μl of FACSLysing solution. After 15 min. of incubation, the lyse-no-wash stained samples were analyzed by FCM utilizing a FACS Calibur. For the two-color PanLeucogating FCM analysis, each two-color reagent-stained blood sample was acquired and analyzed using CellQuest software.

**Novel flow cytometric immunophenotyping method**

In the present study, two new immunophenotyping methods were tested: 1) A four-color FCM method employing a combination of biotin-streptavidin (BS) labeled with APC (BS-APC) and a TriTEST reagent using 52 HIV-infected blood samples. 2) The three-color FCM method based on the use of BS-FITC and the CD45-assisted PanLeucogating FCM approach using CD45-PerCP and CD4-PE on 64 blood samples from HIV-infected patients.

In the four-color FCM staining method, 50 μl of the BS-APC labeled mixture of 4 different WBC samples were mixed with 20 μl of the three-color monoclonal antibody reagent or TriTEST reagent in polystyrene tubes. All tubes were gently mixed and incubated at room temperature for 15 min in the dark. After washing once with PBS, the cell pellets were re-suspended and fixed with 300 μl of 1% paraformaldehyde. The fixed samples were acquired and analyzed by FACS Calibur FCM.

For the three-color FCM method, 50 μl of the BS-FITC labeled mixture of 4 different WBC samples were gently mixed and incubated at room temperature in the dark with 10 μl each of monoclonal antibody to CD45 and CD4 in polystyrene tubes. After a 15 min incubation period, the stained cell mixtures were washed once with PBS, re-suspended and fixed in 300 μl of 1% paraformaldehyde. The fixed samples were acquired and analyzed by FACS Calibur FCM.

**Four-color and three-color flow cytometric analysis**

The cell mixtures stained with BS-APC and the three-color FITC/PE/PerCP conjugated antibodies were analyzed using 4-color CellQuest software by FACS Calibur assisted FCM. The forward scatter (FSC-H) and SSC-H signals were measured using a linear scale (Figure 1A). After acquiring data on 60,000 cells, four regions were established based on the relative intensity of BS-APC signals; SSC/BS-APC⁺ (R1), SSC/BS-APC⁺⁺ (R2), SSC/BS-APC⁺⁺⁺ (R3), and SSC/BS-APC⁺⁺⁺⁺ (R4) cells (Figure 1B). Cells in each of the 4 regions were considered to represent the WBC population from each of the 4 blood samples. Based on the scatter profile, 3 sub-regions were established within each region that represented lymphocytes (SSClow/CD45-PerCPhigh), monocytes (SSCmed/CD45-PerCPintermediate cells) and granulocytes (SSC介/CD45-PerCPlow+) (Figure 1C). Once this region of the first WBC sample was established, the percentage of CD3⁺/CD4⁺ T-lymphocytes in the upper right quadrant was then measured by the CD3-FITC/CD4-PE two-parameter
Figure 1. Representative two-parameter dot plots of 4 different HIV-infected blood samples pre-labeled with and without biotin followed by streptavidin-APC. The mixture of these samples were immunophenotyped for the percentage of CD4+ T-lymphocytes. (A) The FSC/SSC dot plot showing the mixture of 4 different blood samples. (B) Identification of each WBC sample by using SSC/BS-APC, WBCs without biotin are shown in R1, whereas WBCs with 0.001, 0.01 and 0.1 mM of biotin are shown in R2, R3, and R4, respectively. (C) WBCs from R1 stained with three-color monoclonal antibody reagents are detected for their lymphocytes, monocytes and granulocytes using SSC/CD45-PerCP; cells in the gate are lymphocytes. (D) Phenotypic identification of CD3+/CD4+ T-lymphocytes (upper quadrant) from the gate in C is enumerated using quadrant analysis. (E) and (F) Identification lymphocytes and CD3+/CD4+ T-lymphocytes of biotinylated WBCs from R2. (G) and (H). Identification lymphocytes and CD3+/CD4+ T-lymphocytes of biotinylated WBCs from R3. (I) and (J) Identification of lymphocytes and CD3+/CD4+ T-lymphocytes among biotinylated WBCs from R4.
dot-plot (Figure 1D). The percentage of CD3+/CD4+ T-lymphocytes from the other 3 WBC samples (R2, R3, and R4) were also similarly obtained using the CD45+ gating approach (Figure 1E – 1J).

For the three-color FCM analysis, the premixed two-color (CD45-PerCP/CD4-PE) and BS-FITC WBC from 4 different blood samples were analyzed using FACSCalibur assisted FCM by means of the three-color CellQuest software. As in the four-color FCM analysis, a total of 60,000 cells were acquired based on their FSC/SSC profile (Figure 2A). The 4 regions were set to separate each WBC population by their SSC/BS-FITC” (R1), SSC/BS-FITC”” (R2), SSC/BS-FITC+++ (R3), and SSC/BS-FITC++++ (R4) cells, respectively (Figure 2B). For each region, lymphocytes were identified and gated by setting a sub-region denoted as SSC^low/CD45^PerCP^high+ (R5, Figure 2C). Lymphocytes gated within this region were further analyzed for the frequency of CD4-PE expressing cells with SSC^low profile (Figure 2D). CD4+ T-lymphocytes were easily distinguished from non-CD4 T-lymphocytes and thus no isotype control was needed in this gating protocol. A region (R6) defining CD4+ T-lymphocytes was drawn on this SSC^low/CD4^peak plot and the percentage of CD4+ T-lymphocytes was then obtained as a proportion of total R5 lymphocytes. The same approach was also applied to the other 3 WBC populations (Figure 2E – 2J).

Quality control
To ensure quality control of the combination of monoclonal antibody reagents used and the instrument performance, the same batch of reagents was used throughout the study. The FCM photomultiplier tube voltages, sensitivity, and fluorescent compensation settings were optimized prior to sample acquisition and analysis using Calibrite™ beads (BDB).

Statistical analysis
The percentage of CD4+ T-lymphocyte values obtained by the new FCM methods were compared to the standard three-color and the two-color PanLeuco gating methods by linear regression analysis and coefficient of determination (r^2) using StatView™ for Window version 5.0 (SAS Institute Inc., CA). The Bland-Altman statistical bias method was used to determine the level of agreement between the results obtained using the new system and those obtained by the other two systems. The mean percent difference between the two methods (the bias) and the limits of agreement (LOA; equivalent to the mean difference ± 2.0 SD) were then calculated. Statistical significance was considered at p ≤ 0.05. Percent similarity between the results obtained by the new FCM method and the other two previously established methods was also performed by taking the average between the new FCM method and each of the established methods and dividing it by the values obtained by the established method multiplied by 100. Calculation of a coefficient of variation (CV) was carried out and used to define agreement between each data set.

Results
Fluorescence characteristics of biotin-streptavidin-fluorochrome labeled WBCs
White blood cells labeled with biotin and counter stained with streptavidin fluorochrome showed that essentially all of the biotinylated WBCs had positive fluorescence. White blood cells isolated from 3 different unrelated donors were labeled with various concentrations of biotin (0.001, 0.01 and 0.1 mM), respectively. The fluorescence intensity of each WBC population that reacted with subsequent BS-fluorochrome increased proportionally with the amount of biotin (Figure 3B, 3C and 3D). In contrast, as expected, non-biotinylated labeled WBCs showed no or low fluorescent signal following staining with streptavidin-fluorochrome (Figure 3A). The fluorescence characteristics (SSC/BS-fluorescence) of WBC populations were easily detectable over this range of biotin concentration which appeared stable throughout the period of analysis for a period of at least 24 h (data not shown). It should be noted that labeling of WBCs with freshly prepared biotin as compared with previously prepared and stored biotin showed higher fluorescent intensity when stained with streptavidin-fluorochrome. However, use of biotin > 0.1 mM produced off-scale expression of fluorescence (>10^4 arbitrary unit on the logarithmic biotin following incubation with streptavidin-fluorochrome. Thus, WBCs treated with biotin within the range of concentrations of 0.001 – 0.1 mM fresh biotin were used to ensure that WBCs were biotinylated and that acceptable SSC-H/BS-fluorochrome regions were drawn to separate each WBC population.
Affordable CD4 Testing by Flow Cytometry

scale) and failed to show distinct patterns of fluorescence from cells incubated with 0.1 mM biotin followed by streptavidin-FITC. The mixture of these samples were immunophenotyped for the percentage of CD4$^+$ T-lymphocytes. (A) The FSC/SSC dot plot showed the mixture of 4 different blood samples. (B) Identification of each WBC sample by using SSC/BS-FITC; WBCs without biotin are shown in R1, whereas WBCs with 0.001, 0.01 and 0.1 mM of biotin are shown in R2, R3, and R4, respectively. (C) WBCs from R1 stained with two-color PanLeucogating monoclonal antibody reagents are detected for their lymphocytes (R5), monocytes and granulocytes using SSC/CD45-PerCP; cells in the R5 gate are lymphocytes. (D) Phenotypic identification of CD45$^+$CD4$^+$ T-lymphocytes (R6) from the R5 gate is enumerated using SSC/CD4-PE. (E) and (F) Identification of lymphocytes (R7) and CD45$^+$CD4$^+$ T-lymphocytes (R8) among biotinylated WBCs from R2. (G) and (H) Identification lymphocytes (R9) and CD45$^+$CD4$^+$ T-lymphocytes (R10) of biotinylated WBCs from R3. (I) and (J) Identification of lymphocytes (R11) and CD45$^+$CD4$^+$ T-lymphocytes (R12) among biotinylated WBCs from R4.

Figure 2. Representative two-parameter dot plots of 4 different HIV-infected blood samples pre-labeled with and without biotin followed by streptavidin-FITC.
Effect of biotin on the frequency of CD4+ T-lymphocyte determination

A cell mixture containing WBCs from 3 different unrelated HIV-infected patients pre-labeled with BS-fluorochrome and mixed with non-biotinylated WBC from the fourth HIV-infected patient were immunophenotypically stained with the three-color monoclonal antibody reagent or the two-color PanLeucogating reagent and analyzed as described above. Data obtained on the frequency of CD4+ T-lymphocytes in aliquots of whole blood from each of the 4 HIV-infected patients stained individually (one tube) or following the mixing of a separate aliquot of sample from the same 4 HIV-infected patients (4 tubes) are depicted in Figure 4. As seen, there was no detectable difference in the frequency of CD4+ T-lymphocytes noted either when they were stained with the standard three-color TriTEST/MultiSET method or with the two-color PanLeucogating. In addition, there was also no significant difference between the percentage of CD4+ T-lymphocyte values obtained on aliquots of the biotinylated WBCs kept at 4°C for 24 hr and then stained and analyzed and those obtained from aliquots that were stained immediately with streptavidin-fluorochrome and using our standard immunophenotypic staining protocol (data not shown).

Evaluation of the new flow cytometric method

Data obtained from blood samples from a total of 52 HIV-infected patients using FACSCalibur and our newly defined biotinylation method and those obtained using the standard FCM method that included the three-color monoclonal antibody or TriTEST reagents was analyzed for statistically significant differences as shown in Figure 5A. The new FCM method correlated well with the standard three-color FCM method with $r^2 = 0.95$, $y = 0.98x-0.21$, $p < 0.0001$. Figure 5B shows a Bland-Altman plot comparing percent CD4+ T-lymphocyte values from these two methods. The data showed a minimal overall bias of -0.53% and LOA (mean ± 2SD) of -5.22% to +4.16% (the new FCM biotinylation method vs. the standard three-color FCM method) with a percentage similarity of 99.28 and a percent CV of 8.22 (Figure 5C).

When comparisons were made between the percent CD4+ T-lymphocyte values obtained from the new FCM method with those from the two-color PanLeucogating method, the overall correlation coefficient for percent CD4+ T-lymphocyte values from the entire samples was highly significant ($r^2 = 0.97$, $y = 1.04x+0.87$, $p < 0.0001$, Figure 5D). The Bland-Altman plots of the comparison between the two-color PanLeucogating method and those from the new FCM method gave low biases of -0.22% (LOA of -3.42% to +2.98%, Figure 5E). Similarly, there was also a high percentage similarity of 98.15 with a percentage CV of 6.25 (Figure 5F) for the two FCM methods. These data indicate that the new FCM method yields comparable values with an excellent agreement with data obtained using the standard three-color FCM method and the two-color PanLeucogating method.
Figure 4. Comparison of percentage of CD4+ T-lymphocyte values from 4 HIV-infected blood samples analyzed by the new FCM method (one tube) and the standard three-color FCM method (four tubes).

Discussion

In most resource-limited settings, the number of CD4+ T-lymphocytes remains a useful guide for clinical decision-making throughout the course of HIV/AIDS. 2-5 FCM immunophenotyping guidelines rely on detecting specific cell-surface antigen (e.g. CD45, CD4) with the aid of specific monoclonal antibodies that have been labeled with a variety of fluorochromes. In addition to meeting the FCM quality control criteria mentioned in these guidelines, CD4 testing must also be affordable and feasible, especially in resource-constrained countries where the health care system is under increasing pressure to operate cost effectively. Our new method potentially satisfies most of these criteria and was the focus of this study. It is cheaper than the standard three-color and the two-color PanLeucogating methods, as it requires only one monoclonal antibody reagent for the analysis of 4 blood samples. Thus the estimated cost of CD4 testing for one sample is only one-fourth (US$ 4 – 4.50) of the standard reagent cost of US$ 15-18. This cost includes the use of biotin, monoclonal antibody reagents and fluorochrome conjugated streptavidin.

This new FCM method was evaluated for its accuracy and compared to values obtained using the standard three-color FCM and the two-color PanLeucogating methods, since these two methods are widely accessible in many countries. The results from our new FCM method correlated highly ($r^2 \geq 0.95$) with those two methods. The overall bias for CD4+ T-lymphocyte values was 0.53% and 0.22% when the new method was compared with the two previously established FCM methods. These biases will result in CD4+ T-lymphocyte enumeration that are only 0.27% and 0.12% lower when the new method is used, which is unlikely to adversely influence clinical decision making or monitoring of HIV-infected patients.

Biotinylation of WBCs at concentrations within the 0.001 – 0.1 mM range showed that all WBCs were labeled and gave positive fluorescence in a dose-dependent fashion while non-biotinylated controls exhibited no detectable fluorescent signal after streptavidin-fluorochrome counter staining, suggesting that WBCs do not normally have biotin bound to their surface. However, in some BS-fluorochrome stained WBC samples, a substantial proportion of the biotinylated lymphocyte population appeared to form two fluorescent clusters when they were incubated with streptavidin-fluorochrome, particularly at the biotin concentration of 0.1 mM. It is possible that some lymphocytes may have been over stained by the high concentration of biotin. Nonetheless, this phenomenon does not affect immunophenotyping results as long as the region is gated to cover these two BS-fluorochrome stained lymphocyte clusters. One concern with this new FCM method was the stability with which the bitoin would remain bound to the WBCs prior to staining with streptavidin-fluorochrome. Our results show that the fluorescent intensities of biotinylated WBCs at these various biotin concentrations were similar following the addition of the streptavidin-fluorochrome either initially or following 24 h storage at 4°C. Similar long-term stability was also observed when BS-fluorochrome stained WBCs were kept in 4°C for 24 hr prior to three-color and two-color FCM immunophenotyping.

Since this new FCM method simultaneously determines percent CD4+ T-lymphocytes in 4 blood samples, acquiring enough WBCs in particular the lymphocytes from each of these 4 blood samples is therefore essential. This new method may be problematic if the samples are drawn from leucopenia patients or patients with very low lymphocyte counts which may generate statistical errors in determining accurate percent CD4+ T-lymphocytes. Moreover, setting gates to distinguish low number of lymphocytes can be difficult. It is therefore recommended that data on a total leucocyte count of at least 60,000 events is acquired as this number will allow sufficient number of
lymphocytes from each individual blood sample to be gated and analyzed. In our experience, one or two leucopenic or lymphopenic blood samples are not the problem, but if all 4 blood samples are leucopenia or lymphopenia, then one or two blood samples should be replaced by new blood samples with normal or high leucocyte counts. Although our new FCM method is reliable and represents an affordable alternative to the existing three-color and the two-color PanLeucohgating methods, it has to be analyzed using the manual BDB CellQuest or Beckman Coulter EPIC XL System II™ software, a versatile general purpose program that requires the operator to adjust the electronics and set up conditions for data acquisition, analysis, and storage. This is in contrast to the three-color BDB MultiSET software that provides autogating facility to support automated operation that most laboratory technicians prefer. Another drawback is that this new FCM method requires more pipetting steps to complete CD4 testing which will not only cause some inconveniences to the operator but also will potentially impact the safety of laboratory staff, given the highly infectious nature of the virus.

In conclusion, we have developed a novel FCM method, which employs the use of one monoclonal antibody reagent test for simultaneously determining the percent CD4\(^+\) T-lymphocytes in the mixture of 4 different blood samples pre-labeled with biotin. Given the perishable nature and relatively high cost of the reagents, it is therefore more cost-effective to have this new FCM method in resource-limited countries.

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