Low Cost CD4 Enumeration Using Generic Monoclonal Antibody Reagents and a Two-Color User-Defined MultiSET™ Protocol

Kovit Pattanapanyasat,1* Hla Shain,2 Varipin Prasertsilpa,2 Egarit Noursri,1 Surada Lerdwana,1 and Achara Eksaengsri2

1Center of Excellence for Flow Cytometry, Office for Research and Development, Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
2Research and Development Institute, Government Pharmaceutical Organization, Ministry of Public Health, Bangkok, Thailand

Background: The standard three-tube, three-color flow cytometric method utilizing the TriTEST™ reagents in conjunction with the MultiSET™ software commonly used in most laboratories in Thailand for CD4 enumeration is expensive and thus unavailable to most HIV-infected patients. A more affordable method, i.e., the PanLeuco gating protocol using only two monoclonal antibody reagents, has been described but requires the use of the CellIQUEST™ software that does not have automatic gating and reporting facilities. We describe a simple protocol that utilizes a two-color user-defined protocol with the automated MultiSET™ software for the acquisition, analysis, and reporting of CD4 results.

Methods: A two-color user-defined protocol was set up following instructions in the Becton Dickinson Biosciences MultiSET™ manual, adhering strictly to the information regarding the Gate and Attractor Hierarchy for analyzing various reagent combinations. This simple two-color user-defined MultiSET™ software was evaluated using generic monoclonal reagents in comparison with the standard TriTEST™/MultiSET™ protocol.

Results: The two-color user-defined MultiSET™ software is easy to use. It requires only modification of the original MultiSET™ program and the results obtained are comparable with those derived from the standard TriTEST™/MultiSET™ protocol.

Conclusion: The use of this easy and reliable two-color user-defined MultiSET™ protocol represents an affordable alternative to CD4 testing in resource-poor settings. © 2006 International Society for Analytical Cytology

Key terms: acquired immunodeficiency syndrome; CD4 testing; flow cytometry; human immunodeficiency virus; PanLeuco gating

Because of the pioneering efforts of the Afford CD4 group of collaborating scientists, resource poor countries with access to flow cytometers now have the choice of more affordable and simpler protocols for CD4 enumeration [i.e. the PanLeuco gating (PLG) method (1) and the Primary CD4 gating concept (2)], which coupled with the use of generic monoclonal antibody reagents (3) will considerably reduce the economic burden brought about by the HIV-AIDS epidemic. In Thailand, the three-tube three-color flow cytometric immunophenotyping technique (4–6) (CD3/CD4/CD45 for CD4+ T lymphocyte counts and CD3/CD8/CD45 for CD8+ T lymphocyte counts) utilizing the TriTEST™ reagents [Becton Dickinson Biosciences (BDB), San Jose, CA] and the MultiSET™ software program (BDB) is commonly used in most regional hospitals. The cost of CD4 testing remains high and thus beyond the reach of most HIV-AIDS patients. During the past few years, several groups including our own have investigated the simpler protocols (PLG method and primary CD4 gating) and demonstrated excellent agreement between these methods and the standard predicate methods (2,3,7–10). The PLG method requires only two lymphocyte markers (CD45-FITC/CD4-PE) and when combined with the use of generic reagents can reduce the cost of CD4 enumeration without...
sacrificing accuracy and precision. We have recently validated the PLG method for CD4 enumeration in a multicenter trial in Thailand (11). Our results demonstrate that CD4 enumeration by PLG protocol is reliable and represent an affordable alternative to HIV monitoring in resource-poor settings. However, in contrast to the TriTEST™/MultiSET™ software system that provides autogating facility to support automated operation, the PLG protocol runs on the CellQUEST™ (BDB), a versatile general purpose program that requires the operator to adjust the electronics and set up conditions for data acquisition, storage, and analysis (i.e. creation of regions and setting of markers). Some operator training and experience are obviously required for using the CellQUEST™ software. In addition, unlike the MultiSET™ program that provides an automated reporting system capable of producing the Laboratory report, the Physician report, the Summary report, and the Export report, the CellQUEST™ program requires the operator to manually calculate and then transfer the results to another software program for printing in a format requested by the collaborating physician. There is obviously room for clerical errors during the transfer of data from one program to another. For the reasons mentioned, most laboratory technicians in Thailand prefer to use the TriTEST™/MultiSET™ protocol rather than the PLG/CellQUEST™ protocol despite the considerable savings that result from using the latter method. Faced with this dilemma, a suggestion by one member of our group led us to seriously consider putting together a protocol that would combine some of the best features of the two methods mentioned above (i.e. the use of only two markers to define the CD4+ lymphocytes (PLG/CellQUEST™ system) and the automated acquisition, analysis, and reporting of results (TriTEST™/MultiSET™ system).

MATERIALS AND METHODS

Patients and Blood Samples

Peripheral blood samples from 67 HIV-seropositive patients were evaluated in this study. Two milliliters of venous blood were collected by venipuncture into K$_2$EDTA-containing tubes and processed for immunophenotyping within 6 h. One aliquot of EDTA blood from each sample was also sent for complete blood count using Sysmex DF3000 hematology analyzer (TOA Medical Electronics, Kobe, Japan). All blood samples were from routine clinical specimens taken and tested at the Department of Immunology, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand. HIV-1 infections were diagnosed by serologic testing with confirmation by two other different serologic methods. This study was approved by the Ethical Committee of the Faculty of Medicine, Siriraj Hospital.

Flow Cytometric Analysis

The TriTEST™ three-color monoclonal antibodies (BDB) used in this study were directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP) of CD3-FITC/CD4-PE/CD45-PerCP, and used for the predicate method. The generic monoclonal antibody reagents (CD45[2D1]-FITC and CD4[RFT4]-PE for two-color CD4 testing) were obtained from Professor George Janossy of the Royal Free and University College Medical School (London, UK).

Immunophenotypic staining of peripheral blood samples were performed by adding 20 μl of TriTEST™ three-color reagents or generic monoclonal antibodies together with 100 μl of whole blood into 12 × 75 mm$^2$ Falcon polystyrene tubes. The optimal working concentration of these generic reagents was predetermined from titration of the monoclonal antibodies with normal leucocytes and their fluorescence was determined by flow cytometer. All tubes were gently mixed and incubated for 20 min at room temperature in the dark before adding 450 μl of FACS Lysing Solution™ (BDB). After 15 min of incubation, the lye-no-wash stained samples were analyzed with flow cytometer.

The Two-Color User-Defined MultiSET™ Protocol

The MultiSET™ software is designed for acquiring and analyzing samples prepared with one-, two-, three-, and four-color lyse-wash or lye-no-wash user-defined reagents. In setting up this user-defined protocol, the manufacturer's information (12) regarding the Gate and Attractor Hierarchy for BDB-Defined TriTEST™ and MultiTEST™ Reagents, which describes the subset order and plots that the MultiSET™ software uses when analyzing various reagents was adhered to as closely as possible. The setting up of the two-color user-defined protocol is given below.

(A). Identify populations of interest.
Example reagent: CD45-FITC/CD4-PE
Example populations: Lymphs, CD45+, CD45+CD4+, CD45+CD4+, CD45−

(B). Identify the hierarchy.
Example hierarchy
CD45+
CD45+CD4−
CD45+CD4+
CD45−

(C). Translate identified hierarchy to plot parameters for expert gate or attractor.
Example:

<table>
<thead>
<tr>
<th>Population</th>
<th>Plots</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+</td>
<td>FL1 x SSC</td>
</tr>
<tr>
<td>CD45+CD4−</td>
<td>FL1 x FL2</td>
</tr>
<tr>
<td>CD45+CD4+</td>
<td>FL1 x FL2</td>
</tr>
<tr>
<td>CD4+</td>
<td>FL2 x SSC*</td>
</tr>
</tbody>
</table>

*This was added to replace the FL1 x SSC (CD3-FITC/SSC) used in the TriTEST™ protocol.

With this example in mind, choose Reagent Tools from the Tools menu and proceed with the following.
A. Reagent Tools
(1) Choose Reagent Type from the pop up menu.
(2) Choose the reagent you want to copy (i.e. 3/4/45) from the Reagent pop up menu.
(3) Click “Copy Reagent.”
(4) Enter the name of reagent (i.e. CD45/CD4) in the dialog that appears and click OK. The new reagent name with UD appended is added to the Reagents List.
(5) Complete the FL parameter Info. Type CD45 for FL1 antigen and FITC for fluorochrome.
Type CD3 for FL2 antigen and PE for fluorochrome.*
Type CD4 for FL3 antigen and PerCP for fluorochrome.*
(The MultiSET™ software will not accept any attempt by the user to omit the FL3 parameter.) This information is stored as axis labels in data files and displayed at the Acquisition, Lab Report, and Manual Gate views.
(6) Select the checkbox to “Apply Expert Gate.”
(7) Identify the Acquisition Target Info. Choose “lymph” from the pop up menu.
(8) Enter a number (i.e. 5,000) for Min Events to Acquire field.
(9) Define Population Colors/Names. Enter “lymph” in the Expert Gate Field. Allocate appropriate colors to each cell population, i.e. each of CD45+ (red), CD45+CD4− (sky blue), CD45+CD4+ (orange), CD45− (blue). Click OK.
(10) Edit Subset Results
(a) Click Edit Subset Results at the Reagents Tools view.
(b) Use the Population pop up menu to choose the attractor populations you want to select results for (i.e. CD45+ and CD45+CD4+).
(c) Select the Report Population Results checkbox for the chosen attractor population. When CD45+ is selected click Membership%, and for CD45+CD4+ click Ancestor%.
(d) Select “None” in the box below if absolute counts are not required.
(e) Click OK to return to the Reagent Tools view.
(11) Defining Dot Plot Info.
(a) Click Dot Plot Info at the Reagent Tools view.
(b) Click “3” # Plots radio button to choose the number of plots to display.
(c) Choose the X and Y parameters for each plot: Plot 1 FL1 (X parameter) × SSC (Y parameter)
Plot 2 FL2 (X parameter) × SSC (Y parameter)
Plot 3 FL1 (X parameter) × FL2 (Y parameter)
(d) Select the Gate checkboxes for the plots you want to gate. Select the Gate checkbox for FL1 × FL2 and choose “CD45+” from the population pop up menu.
(e) Click OK to return to Reagent Tools.
(f) Click OK in the Reagent Tools view to accept all entries.

B. Creating a new Panel for the new reagent combination.
(1) From the Tools menu choose Panel Tools.
(2) To create a new panel, click “Add Panel.”
(3) Enter a unique name (i.e. CD4 Two-Color) in the dialog that appears and click “Add.”
(4) Choose “User Defined” from the Reagent Type pop up menu.
(5) Choose “CD45/CD4 UD” that appears on the Reagent List and click “Move” to add the reagent to the new panel.
(6) Click OK to record all changes to Panel Tools.

The rest of the procedure (i.e. entering sample information, choosing instrument settings and acquisition) are carried out as for running the TriTEST™ program. Some important points to take note of, include the following:

1. The BDB-defined reagent for the TriTEST™/MultiSET™ system is CD45-PerCP and the expert gate is applied in FL3 × SSC. This is the reason for FL3 being chosen as the threshold parameter with a default value of 300. The threshold parameter must be changed from FL3 to FL1, since the CD4 two-color protocol uses CD45-FITC instead of CD45-PerCP. We have found that using a FL1 threshold value of 300 gives us excellent results.

2. Some adjustment of the attractors for the plot FL1 (CD45-FITC) × FL2 (CD4-PE) may be required. After acquisition, click “Manual Gate” and manually adjust the attractors for CD45+CD4+ (i.e. orange) and CD45+CD4− (i.e. sky blue) populations, before clicking “Analyze.”

The TriTEST™/MultiSET™ protocol was carried out according to the manufacturer’s instruction. For the two-color user-defined MultiSET™ protocol using CD45-FITC/CD4-PE generic reagents, the lymphocytes (SSC low/CD45-FITC+++ cells) were automatically gated as described above following which the modified software automatically determined the percentages of the CD45+CD4+ lymphocytes. Absolute CD4 counts were derived from absolute lymphocyte counts from the hematology analyzer multiplied by % CD4 from the flow cytometer.
Statistical Analysis

Results of % CD4 and absolute CD4 lymphocyte counts obtained by the two methods were compared by linear regression analysis using StatView (Brainpower, Calabasas, CA) and also by Bland–Altman analysis (13).

RESULTS AND DISCUSSION

The PLG method is based on a combination of primary CD45 gating (i.e. CD45/SSC) and primary CD4 gating (i.e. CD4/SSC). The PLG dual platform method is unique in employing CD45/SSC to gate all leucocytes (since the method uses leucocytes as the common denominator between the flow cytometer and the hematology analyzer), but it can also be used to gate lymphocytes if % CD4 among lymphocyte values is required (1). The TriTEST™/MultiSET™ protocol also employs CD45/SSC gating to separate lymphocytes from monocytes and granulocytes. The two methods differ in the gating strategy used to identify the CD4⁺ T-lymphocytes. The TriTEST™/MultiSET™ protocol uses a CD3, whereas the PLG method uses only CD4 expression and side scatter to define CD4⁺ lymphocytes. The two methods employ a similar initial gating step (i.e. CD45+++/SSC) for lymphocyte identification. This together with the manufacturer’s claim that the BDB MultiSET™ software is capable of acquiring and analyzing samples prepared with one, two-, three-, and four-color user-defined reagents provided the rationale for our two-color user-defined MultiSET™ protocol. The dot plots of a representative sample obtained by using the two protocols are shown in Figure 1. It will be noted that a gate for the lymphocytes (i.e. green) as well as two attractors (i.e. red and blue) can be seen in the FL1 (CD45-FITC)/SSC plot in Figure 1 (lower panel) (two-color user-defined MultiSET™ protocol), whereas in the FL3 (CD45-PerCP)/SSC plot derived from the TriTEST™/MultiSET™ protocol only the lymphocytes are gated (i.e. green) (Fig. 1, upper panel). In addition, no attractors defining the CD4⁺ and CD4⁻ lymphocytes can be seen in the FL2 (CD4-PE)/SSC plot in Figure 1 (lower panel). The reasons are given below. In the original TriTEST™/MultiSET™ protocol, the lymphocytes are first differentiated on the basis of granularity (i.e. SSC) and CD45 intensity (CD45-PerCP representing FL3). The gated lymphocytes are then differentiated into CD3⁺ lymphocytes (red attractor) and CD3− lymphocytes (blue attractor) in the FL1 (CD3-FITC)/SSC plot, and CD3⁺CD4⁺ T-lymphocytes (orange attractor) and CD3⁺CD4⁻ T-lymphocytes (sky blue attractor) in the FL1 (CD3-FITC)/FL2 (CD4-PE) plot (Fig. 1, upper panel). The user-defined reagents used in this study are CD45-FITC and CD4-PE; thus, the FL1 (CD45-FITC)/SSC plot in the modified protocol is recognized by the software program as the plot in which attractors are set to define different cell populations, accounting for the presence of a lymphocyte gate and two attractors. Since the parameters CD4-PE/SSC do not figure in the Gate and Attractor Hierarchy in the original MultiSET™ program, no attractors can be seen in this particular plot derived from the user-defined protocol. However, the CD4-PE/SSC plot shows a clear distinction between the CD45⁺CD4⁺ lymphocytes and the monocytes can be used to check whether the CD45⁺CD4⁺ lymphocyte population has been contaminated by monocytes. Any monocyte contamination can then be eliminated by manual adjustment of the orange attractor in the CD45-FITC/CD4-PE plot. The two-color user-defined MultiSET™ protocol is thus based primarily on the use of FL1 (CD45-FITC+++/SSC) to gate the lymphocytes and FL1 (CD45-FITC)/FL2 (CD4-PE) to identify the CD45⁺CD4⁺ lymphocytes.

In this study, we have compared the % CD4 and absolute CD4 values obtained by the two protocols and found the results to be comparable as the coefficient of determination ($R^2$) values of 0.9902 ($r = 1.0023 x - 0.6552$).
P < 0.0001) and \( R^2 = 0.9952 \) (\( y = 0.9772x - 5.5237 \), \( P < 0.0001 \)), respectively (Figs. 2A and 2B). Bland–Altman bias plots comparing the two protocols for both % CD4 (Fig. 2C) and absolute CD4 values (Fig. 2D) showed excellent agreement. The % CD4 lymphocytes showed a minimum overall bias of +0.6%, whereas for absolute CD4 lymphocyte counts, the overall bias was only 15.1 cells/µL (limits of agreement -26.2 to +56.4 cells/µL). A multicenter trial comparing the two protocols is currently underway to confirm these findings and to determine whether the two-color user-defined MultiSET™ protocol can be used as an alternative to the standard TriTEST™/MultiSET™ protocol. We have described the dual platform protocol, since this is the method in common use in Thailand. This two-color user defined method can also be used on a single platform if TruCount™ tubes or beads are used.

The PLG method has demonstrated that the employment of only two markers using primary CD45 and primary CD4 gating allows the identification of CD4⁺ lymphocytes without the requirement of an additional CD3 marker, an important cost issue in resource poor countries. Here, we have demonstrated that the CD3 made very little difference to the determination of % CD4 among lymphocytes when the two-color user-defined method was compared to the TriTEST™/MultiSET™ protocol. Although, consistency checks would be facilitated by its inclusion (1,2,7,14). The two-color user-defined MultiSET™ protocol, which requires only a simple modification of the MultiSET™ software, combined with the use of generic monoclonal antibody reagents will not only reduce errors arising from manual gating and manual reporting of results associated with the CellQUEST™ software, thus leading to increased laboratory throughput, but also reduce the cost of CD4 testing, thereby allowing more HIV/AIDS patients access to CD4 monitoring in resource poor countries.

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LITERATURE CITED


