Affordable CD4\(^+\)-T-Cell Counting by Flow Cytometry: CD45 Gating for Volumetric Analysis

George Janossy,1* Ilesh V. Jani,1 Nicholas J. Bradley,1 Arsene Bikoue,1 Tim Pitfield,1 and Debbie K. Glencross2

HIV Immunology, Department of Immunology and Molecular Pathology, Royal Free and University College Medical School, London, United Kingdom,1 and Department of Molecular Medicine and Hematology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa2

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The flow cytometers that are currently supported by industry provide accurate CD4\(^+\)-T-cell counts for monitoring human immunodeficiency virus disease but remain unaffordable for routine service work under resource-poor conditions. We therefore combined volumetric flow cytometry (measuring absolute lymphocyte counts in unit volumes of blood) and simpler protocols with generic monoclonal antibodies (MAbs) to increase cost efficiency. Volumetric absolute counts were generated using CD45/CD4 and CD45/CD8 MAb combinations in two parallel tubes. The percentage values for the various subsets were also determined within the leukocyte and lymphocyte populations utilizing a fully automated protocol. The levels of agreement between the newly developed method and the present industry standards, including both volumetric and bead-based systems using a full MAb panel for subset analysis, were tested by Bland-Altman analyses. The limits of agreement for CD4 counts generated by the volumetric methods using either CD45/CD4 (in a single tube) or the full Trio MAb panel (in three tubes) on the CytorunAbsolute flow cytometer were between -29 and +46 cells/mm\(^3\) with very little bias for CD4 counts (in favor of the Trio method: +8 CD4\(^+\) lymphocytes/mm\(^3\); 0.38% of lymphocytes). The limits of agreement for absolute CD4 counts yielded by the volumetric CD45/CD4 method and the bead-based method were between -118 and +98 cells/mm\(^3\), again with a negligible bias (-10 CD4\(^+\) lymphocytes/mm\(^3\)). In the volumetric method using CD45/CD8, the strongly CD8\(^+\) cells were gated and the levels of agreement with the full Trio showed a minor bias (in favor of the Trio; +40 CD8\(^+\) cells/mm\(^3\); 5.2% of lymphocytes) without a significant influence on CD4/CD8 ratios. One trained flow cytometrist was able to process 300 to 400 stained tubes per day. This workload extrapolates to a throughput of >30,000 samples per year if both CD45/CD4 and CD45/CD8 stainings are performed for each patient or a throughput of >60,000 samples if only CD45/CD4 counts are tested in a single tube. Thus, on the basis of the high efficiency and excellent agreement with the present industry standards, volumetric flow cytometers with automated gating protocols and autobiosamplers, complemented by generic CD45, CD4, and CD8 MAbs used in two-color immunofluorescence, represent the most suitable arrangements for large regional laboratories in resource-poor settings.

Dedicated flow cytometers are designed to enumerate the absolute numbers and percentages of lymphocyte populations, such as subsets of T cells, B cells, and NK cells. In the clinical service for monitoring human immunodeficiency virus (HIV) disease, the primary aim is to deliver absolute CD4\(^+\)-T-cell counts, and this is achieved with a remarkably high level of precision (9, 24, 26, 29, 30). Nevertheless, the various cytometric systems differ in complexity (9). It has recently been documented that routine CD4-T-cell enumeration can be simplified without compromising quality (13, 18, 32), leading to cost-effective services for patients who receive generic antiretroviral drugs in resource-poor settings (Fig. 1).

Among the flow cytometers, dedicated instruments operating as “single platforms” are preferred due to their convenience and accuracy (9, 24, 26, 29, 30). These single platforms are based either on a volumetric principle by counting CD4\(^+\) T cells in a unit volume of blood (24, 26) or on the concept of adding known numbers of fluorospheres, or “microbeads,” to each sample (29, 30). These beads are, however, precision products that can increase running costs. Consequently, services handling large numbers of samples had to revert to “double platforms” operating a panleucogating strategy with CD45 monoclonal antibody (MAb) to secure a much less expensive but still accurate mode of operation (13, 19). Indeed, the CD45-based gating, an example of the “heterogeneous” gating strategy, is a more reliable protocol (12, 13, 20, 25) when used with autogating in aging samples (4, 13, 25) than the conventional gating strategies that utilize morphological scatter gates (27, 31).

Despite the present interest in improving the efficacy of routine flow cytometry (Fig. 1), the performance of volumetric flow cytometric systems (10, 18, 24) operating with CD45-based gating (4, 13) and generic MAbs has not yet been assessed. We have therefore investigated the following topics: (i) the agreement between the results of CD45/CD4 staining using simple panleucogating (13) on volumetric single platforms and those obtained on the full volumetric (24) and bead-based (30) systems during CD4\(^+\)-T-cell enumeration, including both absolute counts and CD4 percentage values (among leukocytes and lymphocytes); (ii) the increased sample throughput using

* Corresponding author. Mailing address: HIV Immunology, Department of Immunology and Molecular Pathology, Royal Free and University College Medical School, Rowland Hill St., London NW3 2PF, United Kingdom. Phone: 44-20-7830 2349. Fax: 44-20-7431 0879. E-mail: janossy@rfhsm.u-net.com.
| year      | (A) QA programmes | (B) Sample handling | (C) Generic antibodies | (D) Gating methods | (E) Volumetry | (F) Red-diode laser |
|-----------|-------------------|---------------------|------------------------|-------------------|---------------|--------------------|
| 1980-85   | —                 | Lyse-no-wash method (15) | Characterisation of CD45, CD4, CD8 Abs (8, 23) | —                 | —             | —                  |
| 1986-89   | —                 | Directly conjugated MAbs | Generic CD4 MAbs are deposited at NIBSC | Primary CD45 gating for leukocyte differentials (20) | —             | —                  |
| 1990-92   | NEQAS for lymphocyte subsets (1) | —                 | —                      | Primary CD3 gating (22) | —             | —                  |
| 1993-94   | —                 | —                 | —                      | —                 | Red-diode for CD8 | —                  |
| 1995-97   | Stabilized cells for multicentre studies (10) | CD45 gating is more reliable than scatter used on its own (25) | —                      | Powerful volumetric flow cytometer with large biosampler (24) | Detailed concept of red-diode laser cytometry (31) | —                  |
| 1998-99   | QASI launched for Canada & the developing world (21) | TransFix blood stabiliser is launched (2) | The first paper is published about the possibility of cost-effective CD4 enumeration by flow cytometry (32) | —                 | —             | —                  |
| 1999-2000 | NEQAS and NIBSC launch the EuroStandard | TransFix is used for intercontinental sample transfer (17) | More generic MAbs are deposited at the NIBSC and in South Africa and labelled with different fluorochromes (18) | Primary CD4 gating is performed on volumetric flow cytometer using a single CD4 antibody (18) | —             | —                  |
| 2001-02   | QASI and NEQAS collaborate in providing complementary, non-profit QA | —                 | Generic MAbs are introduced in lectures and in comparative studies [this paper] | Panleucogating (CD45/4) performs well on double platforms (13) | High capacity CD45/CD8 testing on volumetric single platform [this paper] | —                  |

FIG. 1. Recent events leading to affordable CD4-T-cell enumeration by flow cytometry. NIBSC, National Institute for Biological Standards and Control; NEQAS, UK National External Quality Assessment Service, Sheffield, United Kingdom; QASI, Quality Assessment & Standardization for Immunology, Ottawa, Canada.
CD45/CD4 staining; (iii) the extension of this protocol to include a second tube for CD45/CD8 staining in order to obtain CD4-plus-CD8 counts and CD4/CD8 ratios; and finally, (iv) the use of volumetric CD45 staining for generating absolute and differential counts for leukocyte subsets (20).

Our study reveals the practical advantages of volumetric two-color flow cytometry with CD45/CD4 and CD45/CD8 staining using generic MAbs. Volumetric cytometers, equipped with biosamplers of high capacity, Microsoft Windows-based autogating software, and reporting systems, efficiently handle 300 to 400 samples during a working day. As many as 15 parameters, including CD4 and CD8 analysis together with hematological leukocyte differentials, can be generated for cost-efficient monitoring of HIV-infected patients in large regional laboratories.

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MATERIALS AND METHODS

Clinical samples. Samples (n = 93) were received for routine immunological diagnosis at an HIV-immunology laboratory and included patients at various stages of HIV infection (Table 1) as part of the routine diagnostic and quality assurance activity at the Royal Free Hospital. No extra specimens from HIV-seropositive patients were required. Twelve additional samples were taken from healthy volunteers 21 to 59 years of age as approved by the Institutional Ethics Committee (Table 1). These whole-blood samples were collected in EDTA and analyzed within 24 h using a “lyse-no-wash” procedure (15, 18). Briefly, in each tube, 10 or 20 µl of a diluted mixture of antibodies was admixed with 50 or 100 µl of whole blood, respectively. After 15 min of incubation at room temperature, 2.0 ml of lysis solution (0.17 M NH4Cl) was added. The samples were counted after a final 15-min incubation (17, 24).

Instrumentation. Absolute lymphocyte subset counting was performed on a Bayer 120 hematology analyzer in the hospital's hematology laboratory as part of the routine service.

Reagents. During the volumetric-control procedure on the Ortho Cytoron absolute, Ortho Trio MABS were used (10, 24). These included three tubes comprising in tube 1 isotype controls (immunoglobulin G1 [IgG1] plus IgG2a-FITC–IgG1 plus IgG2a-PE–IgG2a-PECy5), in tube 2 CD4(OKT4)+FITC–CD8(OKT8)-PE–CD3(OKT3)-PECy5, and in tube 3 CD16(G50)-FITC–CD19(OKB9)-PE–CD3(OKT3)-PECy5 (original clone designations are shown in italics). During the bead-based control procedure on the FACSCalibur, TruCOUNT tubes were used in combination with MultiTEST reagents (Becton Dickinson Immunocytometry Systems) to obtain absolute CD4 counts (30). These included CD3(SK7)-FITC–CD8(SK8)-PE–CD45(2D1)-PerCP–CD45(2SK)-APC. The new CD45-based protocol was also based on the volumetric procedure performed on the Ortho Cytoron. Two tubes containing two-color immunofluorescence (IF) reagents were each tested. Tube 1 contained CD5(2D1)-FITC–CD45(2RT)-PE, and tube 2 comprised CD45(2D1)-FITC–CD8(2RT)-PE. These generic reagents are available in unconjugated form from the National Institute for Biological Standards and Control (Potters Bar, United Kingdom).

Gating strategies for CD4 and CD8 enumeration. On the Ortho Cytoron, we employed the Trio reagents and obtained absolute counts for the following cell types: T cells (CD3+, low side scatter), CD4+ T lymphocytes (CD3+ CD4+), CD8+ T lymphocytes (CD3+ CD8+), B cells (CD19+, low side scatter), NK cells (CD3+ CD16+), and total lymphocytes (CD4+ T plus CD19+B plus CD16+ NK cells referred to as Immunosum) (24). Percentage values for CD4+ T lymphocytes (CD4%) were derived as the number of CD4+ T cells divided by the total number of lymphocytes based on the criteria of CD4+, CD3+, and CD8+ cells/Immunosum (10, 18, 24). The CD4/CD8 ratios were calculated as (CD3+ CD4−)(CD3+ CD8+) values. The internal quality control for pipetting errors was based on CD3 replicates using Immunocount II software (10): samples for which the CD3 replicates differed from the average absolute CD3 count by >5% were automatically flagged for further inspection. The event threshold was set to operate on forward scatter. On the FACSCalibur, the gating strategy recommended by the manufacturer was used, with the threshold set for red fluorescence (CD45) in a single tube (30).

For the new protocol, the gating strategy was based on CD45 panleucocytating (17, 19). A threshold was first set for green (CD45) fluorescence, and all WBCs were identified (Fig. 2) using a heterogeneous CD45/SSc dual-parameter histogram (CD45+ to CD45+ in gate A). All WBC events in gate A were then sent to a CD45/SSc histogram, where CD4 T cells were counted (CD4+/SSc− in gate E [Fig. 2]). The same gating strategy was applied for CD8 counting in a second tube. Here, only lymphoid cells with bright CD8 expression were counted as CD8 T cells (CD8+/SSc−) in gate F (18). The CD4/CD8 ratios were calculated as (CD4+−SSc+)/(CD4+−SSc+) values. All these gating strategies were set to operate automatically and printed with all details (Fig. 2). The internal quality control for pipetting errors was based on CD45 WBC replicates using the Immunocount II program. If the CD45 total WBC replicates differed from the average absolute CD45 count by >5%, the samples were flagged. All flagged samples or those where the operator had detected gating irregularities were subsequently reanalyzed.

In the second stage of the analysis, the different CD45 staining intensities among the lymphocyte populations (20) were used to identify lymphocytes (CD45−SSc− in gate B), monocytes (CD45+/SSc− in gate C), and granulocytes (CD45+/SSc− in gate D). Using absolute counting and a two-color IF panel in two parallel tubes, the following 15 parameters were distinguished and stored: (i) total WBC counts, (ii) absolute CD4-T-cell counts, (iii) CD4-T-cell percentage among WBC, (iv) CD4-T-cell percentage among lymphocytes, (v) absolute CD8-T-cell counts, (vi) CD8-T-cell percentage among lymphocytes, (vii) absolute CD4- plus CD8-T-cell counts, (ix) CD4/CD8 ratio, (x) absolute lymphocyte counts, (xi) absolute monocyte counts, (xii) absolute granulocyte counts, (xiii) lymphocyte percentage among WBC, (xiv) monocyte percentage among WBC, and (xv) granulocyte percentage among WBC. In samples where a single tube was analyzed with CD45/CD4, 10 parameters (i to x and x to xv) were recorded.

Data handling and statistical analysis. All results have been recorded in Microsoft Access-based spreadsheets. Following consultations with clinicians, forms were created for reports. Depending on the requests, these could include the simple parameter of absolute CD4 count or all 10 to 15 parameters recorded according to the request.

After we tested whether the differences between the methods were normally distributed (13), Bland-Altman plots (7) were used to investigate the agreement between the results obtained in two different systems, such as the panleucocytating analysis on a volumetric flow cytometer versus a conventional “industry-standard” method. The standard techniques included the volumetric flow cytometer,
**RESULTS**

**CD4\(^+\)**-T-cell enumeration using primary CD45 and CD4 gating. Total lymphocytes were identified by volumetric counting (i) as the sum of T cells, B cells, and NK cells (Immunosum) using Trio MAb (referred to as a full Trio panel) and (ii) as cells with a bright CD45 expression and lymphoid side scatter in the CD45-based protocol. No significant systematic bias was observed between the two methods (bias = −8 lymphocytes/mm\(^3\) [Fig. 3a]).

Next, CD4\(^+\)-T-lymphocyte counts were determined by the full Trio panel and the CD45/CD4-based protocol, i.e., in the presence and absence of a CD3 reagent, respectively. The CD4\(^+\) values among lymphocytes generated by the two methods showed a minimal bias of +0.38% (in favor of the Trio MAb [Fig. 3b]). The absolute CD4-T-cell counts yielded by the two methods also showed excellent agreement (bias = −8 CD4\(^+\) cells/mm\(^3\); limits of agreement, between −29 and +46 CD4 cells/mm\(^3\) [Fig. 3c]).

The agreement between the volumetric absolute CD4-T-cell counts using CD45/CD4 double staining with Panleucogating was also determined. An average bias of −10 CD4 cells/mm\(^3\) was observed with widened limits of agreement (−118 and +98 CD4 cells/mm\(^3\)), similar to the values previously observed between the standard volumetric and bead-based single-platform technologies (13, 18).

**Efficiency of the CD45/CD4 gating protocol on a volumetric system.** After having documented CD4 enumeration using a CD45/CD4-based protocol by volumetric flow cytometer, we assessed the sample throughput of the system. A technician

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**Graph Report**

**Facility:** HIV Immunology, RFUCMS

**Address:** Royal Free Campus, Hampstead London NW3 2PF

**Phone:** 44-20-78302349

**Director:** Prof. G. Janossy.

**Operator:** Dr. I. Jani.

**FCS File Path:** d:\leah\45e4e8

**PID:** 60

**Analysis Date:** Tue Mar 26 17:13:16 2002

**Protocol:** System Protocol (50)

*fully automated autogating protocol using auto-biosampler of 100 tube capacity (100 tubes in each 120 minutes)*

**QA by consultant physician and NEQAS**

*Volumetric setting with Panleucogating*

**Comment:** Normal blood with 924 CD4/sil

*Signed by senior scientist*
FIG. 3. Bland-Altman plots to establish the agreements between the volumetric CD45/CD4 protocol, single tube, and the state-of-the-art single-platform technology. The parameters studied were the total absolute lymphocyte counts (a), CD4-T-cell percentage values among lymphocytes (b), and absolute CD4-T-cell counts (c and d). The standard technologies used were the full lymphocyte subset panel (three tubes) tested with the Ortho Trio panel on the Cytoron Absolute (a, b, and c) and the TruCOUNT bead-based method (one tube) performed on a FACSCalibur (d).
CD8⁻ T cells represented a 10.1% bias throughout the whole range of the T-cell counts (Fig. 5c).

**WBC subset enumeration using CD45-based protocols on a volumetric flow cytometer.** The expression of CD45 antigen is the common feature of all WBCs, and the CD45 staining intensity plus SSC distinguishes lymphocytes, monocytes, and granulocytes (Fig. 2) (20). We investigated the agreement between counts generated by this method and those yielded by a hematology analyzer in the routine hematology laboratory of our institution. The agreements for total WBCs, lymphocytes, and granulocytes were good, with a minimal bias of −136 WBCs/mm³, −70 lymphocytes/mm³, and +78 granulocytes/mm³ (Table 2). However, the agreement for monocyte enumeration was poor. The hematology analyzer underestimated the monocyte counts with a bias of −179 monocytes/mm³. This is a large value, representing 35 to 40% of the total monocyte counts, as already reported for several hematology analyzers (14, 19).

**DISCUSSION**

The need to improve laboratory services for regions of the world where the HIV epidemic threatens to destroy the fabric of life has revitalized efforts to identify the most efficient techniques for monitoring HIV disease. The present changes relate to the common areas of routine immunology and hematology, such as quality assurance, sample processing, and transportation, as well as to the challenges of how to optimally count blood cells (Fig. 1). Immunological methods, based on the specificities and discriminating capacities of MAbs, have recently made an impact by recognizing even minor subsets of functionally divergent blood cells (9, 24, 26, 29, 30). By using flow cytometry, the true power of directly identifying cells by antibodies, as opposed to first investigating merely their morphological features, is now documented, and the strategy of primary immunological gating is widely used (12, 18, 20, 22). The relevant examples include CD45 for leukocytes and their subpopulations (4, 13, 20), CD3 for T cells (22, 25), CD4 for the major T-cell subset and monocytes (17, 18), and CD8 for the minor T-cell subset and some NK cells (18). Reliable total lymphocyte counts have been achieved by the Immunosum technique (24), providing the sum of the immunogated CD3⁺ (T), CD19⁺ (B), and CD16⁺ (NK) cells instead of using only the lymphocytic scatter appearance. The commonly used display on the cytometers is referred to as a heterogeneous, or morphospectral, protocol (23, 27) to show the IF of cells stained with MAAb (on one axis) and the side scatter profile of cells (on the other axis) (Fig. 2).

In our study, we combined immunological CD45 gating with volumetric absolute counting on single platforms in order to introduce a robust method for WBC counting and for enumerating CD4⁺ and CD8⁺ T cells. Our four main findings are as follows.

First, this study confirms our previous work, also performed on volumetric flow cytometers (18), as to the good agreement between the absolute CD4⁺-T-cells counts obtained by direct CD4 gating and by CD4⁺ CD8⁺ coexpression (Fig. 3c). Importantly, however, when we previously used CD4 MAb on its own without CD45, reliable CD4%-(per-lymphocyte values could be obtained only with the constant vigilance of an expe-
rienced operator, who frequently had to manually modify the lymphocyte gates, a time-wasting procedure (18). We have now added CD45 gating to the protocol and report the excellent agreement between lymphocyte counts determined by CD45-side scatter and by the Immunosum method using the full Trio panel (Fig. 3a). Thus, CD45 staining improves the efficiency of the new autogated protocol (Fig. 2), saving effort and technicians’ time. Similarly, this gating strategy shows no bias compared to the bead-based CD4 counts (30) but reveals occasional differences leading to a wider spread (Fig. 3d). This discrepancy might be a bead-related phenomenon, because similar results are seen when CD4 counts obtained by panleucogating on a double platform are compared to counts obtained by the bead-based method (13, 18).

The second conclusion is that the CD45/CD4 protocol on a volumetric cytometer provides an efficient system in which a trained flow cytometrist can run large numbers of tubes per day (>300 samples using CD45/CD4 alone [Table 2]). If two parallel tubes are used with CD45/CD4- and CD45/CD8-double-stained cells, >150 blood samples can be studied. Obviously, such intensive diagnostic activity needs to be supplemented by clerical help and supervisory capacity. Nevertheless,
this capacity illustrates the high efficiency of flow cytometry compared to that of manual methods such as the Dynabeads system (11), where a single assistant can manually handle only 15 to 20 samples per day. A hugely increased workload for CD4-T-cell enumeration is in line with the expected demand generated by the arrival of generic drugs for antiretroviral therapy. The larger regional centers dedicated to nationwide support with organized sample transportation using TransFix blood stabilizers (17) will require this increased service capacity.

The technical efficiency of this technology is directly related to three factors: (i) the fluent operation with a robust autogating process, where only <2 to 4% of samples need attention for regating (see above) (Fig. 2), (ii) the use of an efficient autobiosampler (10, 24), and (iii) a convenient system using a Windows environment and a Microsoft Access database for feedback to the clinicians. Flexible reporting, based on consultation with clinicians, may include only CD4 counts or any of the 15 parameters listed in Materials and Methods.

The third finding of our study is related to the use of CD3,

FIG. 5. Bland-Altman plots (a and b) and the Pollock modification (c) to establish agreements on the Cytoron between the volumetric CD45/CD4-plus-CD45/CD8 two-tube protocol and the standard volumetric method using Trio reagents. The parameters studied were the CD4/CD8 ratios (a) and the sum of the absolute CD4- plus CD8-T-cell counts versus the CD3- T-cell counts (b and c, respectively). In the Pollock modification (c), the differences in total T-cell counts were expressed as percentages of T-cell counts to illustrate the regular underestimation of total CD3- T-cell counts, at a 10% level, by the CD45 protocol. This bias is due to the existence of CD3- CD4- CD8- T lymphoid cells.
the specific T-cell marker. Arguably, CD3 is not required to identify CD4+ T cells (13, 17, 18). However, the CD8–lymphocyte populations are more complex (18) and display CD8 antigen over a wide range (15 × 103 to 140 × 103 CD8 molecules/cell [5]). The CD8– cells include 80 to 92% proper CD8+ CD3+ T cells that display CD8 at a high level (CD8+; 80 × 103 to 140 × 103/cell) and 8 to 20% CD8+ CD3– NK cells that express CD8 at a lower level (CD8–; <80 × 103/cell). It is therefore logical to place a tight gate around the CD8+ population and compare these results with those obtained by counting CD3+–gated CD8 T cells (18). The results described above show that the CD8+– gate underestimates CD3+ CD8+ counts by 5.2% (Fig. 4c). This bias is apparently too modest to influence the CD4/CD8 ratios (bias, −0.05 [Fig. 5a]). An extra advantage of running both CD45/CD4 and CD45/CD8 tubes is the availability of CD4–plus CD8–T-cell counts that disregard the CD3– CD4+ CD8– T cells. We have argued elsewhere that these double-negative T cells represent a functionally different, mostly T-cell receptor αβ-negative subset that should not be included in the total T-cell counts (18).

Finally, Loken et al. (20) have documented the differential expression of CD45 antigen on lymphocytes, granulocytes, and CD14+ monocytes. In our study, the CD45 analysis is combined with volumetric counting in order to generate absolute leukocyte differential counts. These parameters, when defined on hematological counters, can be error prone (3, 19, 33), and the monocyte counts are frequently underestimated (Table 3) (14, 19). On the other hand, the monocyte counts obtained by CD45 gating and carefully confirmed by the CD14 mononcytic marker expression (20) are more accurate. Consequently, the methods described above, in combination with the use of stabilized blood preparations with long shelf lives (1, 13), will assist the establishment of long-awaited quality assurance schemes for leukocyte differentials and absolute counts, which are required to coordinate the performance of the wide variety of different hematologic analyzers.

In conclusion, the present volumetric CD45/CD4 flow cytometry, assisted by more affordable sources of MABs, has wide applicability in the routine laboratories operating in economy-conscious environments. The specification required for the two-color IF plus side scatter used in this study is within the reach of the newly designed, battery-operated, smaller-volumetric-flow cytometers that carry red diodes or other small light sources as the sole source of light excitation (19, 31) and are also capable of performing bead-based enzyme-linked immunoabsorbent assays with the multiplexing technology (19) in the area of the differential diagnosis of infectious diseases (16).

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