Absolute CD4 T-Cell Counting in Resource-Poor Settings

Direct Volumetric Measurements Versus Bead-Based Clinical Flow Cytometry Instruments

Tandakha Ndiaye Dieye, PharmD, PhD, Chris Vereecken,† Abdoul Aziz Diallo, MS,* Pascale Ondoa, MD, PhD,† Papa Alassane Diaw, PharmD,* Makhtar Camara, PharmD,* Farba Karam, PharmD,* Souleymane Mboup, PharmD, PhD,* and Luc Kestens, PhD†*

Summary: Flow cytometry is an accurate but expensive method to determine absolute CD4 cell counts. We compared different methods to measure absolute CD4 counts in blood samples from HIV-infected and uninfected subjects using a research/clinical flow cytometer (FACScan); a dedicated clinical instrument (FACSCount); and a volumetric, mobile, open-system flow cytometer equipped with 3 fluorescence and 2 light scatter detectors (Cyflow SL blue). The FACScan and Cyflow were used as single-platform instruments, but they differ in running cost, which is a central factor for resource-poor settings. Direct volumetric and bead-based CD4 measurements on the Cyflow were compared with 2 bead-based single-platform CD4 measurements on the FACSCount and on FACScan (TruCount) in "Le Dantec" Hospital, Dakar, Senegal, using whole blood samples from 102 HIV^+ and 28 HIV^- subjects. The agreement between the various measurement methods was evaluated by Bland-Altman analysis. Volumetric CD4 measurements on the Cyflow using a no-lyse-no-wash (NLNW) procedure and a lyse-no-wash (LNW) procedure correlated well with each other (R^2 = 0.98) and with CD4 measurements on the FACSCount (R^2 = 0.97) and FACScan (R^2 = 0.97), respectively. Red blood cell lysis had no negative effect on the accuracy of absolute CD4 counting on the Cyflow. An excellent correlation was observed between bead-based CD4 measurements on the Cyflow and CD4 measurements on the FACSCount (R^2 = 0.99) and FACScan (R^2 = 0.99). Rigid internal and external quality control monitoring and adequate training of technicians were considered essential to generate accurate volumetric CD4 measurements on the Cyflow.

Received for publication November 11, 2004; accepted February 13, 2005.

From the *Immunology Unit, Laboratory of Bacteriology-Virology, Cheikh Anta Diop University, Dakar, Senegal; and †Laboratory of Immunology, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium.

Financial support provided by the Belgian Directorate General for International Collaboration (DGIC).

Reprints: Tandakha Ndiaye Dieye, Immunology Unit, Laboratory of Bacteriology-Virology, Le Dantec University Teaching Hospital, Avenue Pasteur, BP 3001, Dakar, Senegal, (e-mail: tndieye@yahoo.co.uk and dieye@cnts-dakar.sn).

Key Words: CD4 count, HIV, affordable flow cytometry

(*J Acquir Immune Defic Syndr* 2005;39:32–37)

As a result of international efforts, highly active antiretroviral therapy (HAART) is becoming more accessible to people living with HIV/AIDS in developing countries. Laboratory markers such as the absolute CD4 cell count are important in deciding when to initiate HAART in HIV-infected subjects.[1] Unfortunately, adequate and affordable laboratory monitoring tools are frequently lacking in resource-limited settings, and efforts should be undertaken to improve their access to laboratory monitoring as well.[2] The "gold standard" technology for CD4 counting, which allows a relatively large throughput of samples, is flow cytometry, but this technology is often too expensive and too sophisticated to be used in poorly equipped hospital laboratories in resource-limited countries. The lack of laboratory monitoring may render treatment less effective and could ultimately jeopardize the HIV/AIDS care program in developing countries. Alternative manual CD4 counting tools, although useful under certain circumstances, are less accurate and do not allow a high sample throughput.[3]

Many clinical flow cytometers only measure CD4 T-cell percentages and have to be used in combination with the absolute lymphocyte count provided by hematologic analyzers to calculate the absolute CD4 T-cell count (dual platform).[4] To avoid the use of a dual platform, expensive precision-made microspheres with a known concentration can be purchased to count the absolute number of CD4 T cells on the same instrument.[5] An example of a dedicated clinical single-platform bead-based CD4 counting instrument used in many developing countries is the FACSCount (Becton Dickinson).[6] The running cost of this instrument is relatively high, however, because expensive, dedicated, bead-based CD4/CD8 reagents; essential internal quality control reagents; and costly preventive maintenance by service engineers are required.

Recently, more affordable and mobile single-platform flow cytometers that do not require dedicated reagents and expensive preventive maintenance contracts have become available. Data are lacking on the performance of this new

generation of volumetric flow cytometers under field conditions and independently from the manufacturer.

The present study was undertaken to evaluate and compare the results of single-platform absolute CD4 counting on a mobile Cyflow SL blue (Partec GmbH, Münster, Germany) instrument, with the results obtained from state-of-the art, single-platform, bead-based CD4 measurements on FACSCount and FACScan (TruCount; Becton Dickinson) instruments.

MATERIALS AND METHODS

Blood Samples

One hundred and two HIV^+ and 28 HIV^- whole blood samples were obtained from outpatients presenting for clinical HIV disease follow-up or routine laboratory examinations at the University Hospital "Le Dantec" in Dakar, Senegal in July 2002. The blood samples were collected in EDTA Vacutainer tubes (Becton Dickinson) and processed within 8 hours after collection in the Laboratory of Bacteriology and Virology of the same hospital.

Immunophenotyping by Flow Cytometry Methods

Flow cytometry analysis was performed on 3 different flow cytometers: (1) a Cyflow SL blue equipped with a 488-nm blue solid-state laser and 5 detectors; (2) a FACSCount, a clinical instrument for CD4 counting using dedicated reagents; and (3) a FACScan.

Direct volumetric CD4 measurements were performed on the Cyflow as follows: 50 μL of whole EDTA blood was pipetted into polystyrene test tubes. Ten microliters of a mixture of monoclonal antibodies containing CD3–fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE), and CD45-peridinin-chlorophyll-protein (PerCP) was added to the blood in the test tubes and left to incubate for 15 minutes at room temperature. Before acquisition, 2 mL of phosphate-buffered saline (PBS) was added to the tube for direct no-lyse-no-wash (NLNW) volumetric CD4 measurements. Alternatively, 2 mL of red blood cell (RBC) lysing solution (Becton Dickinson) was added for direct lyse-no-wash (LNW) volumetric CD4 measurements on the Cyflow. The acquisition and analysis of lysed whole blood were done according to a strategy based on CD45 versus side-scatter (SSC) gating, as initially described by Nicholson et al[7] and later designated panleucogating by Glencross et al.[8] For the analysis of unlysed whole blood, the acquisition trigger was set on CD45 and the bright $CD45^+$ cells were gated. Subsequently, the $CD4^+CD3^+$ cells were gated in the FL1 × FL2 scatterplot. The concentration of CD4 cells per microliter was calculated by multiplying the counted CD4 cells by the dilution factor (41.2 = 50 μL of blood in a final volume of 2060 μL) and dividing the result by 200 (the Cyflow counts cells in a fixed volume of 200 μL).

Using FACSCount reagents, 50 μL of EDTA blood was added to the CD4 FACSCount reagent tubes containing anti-CD3 and anti-CD4 antibodies, sample diluent, and reference beads. According to the routine protocol, the samples were vortexed and incubated for 30 minutes at room temperature. The samples were run on the FACSCount. After analysis on the FACSCount, the remaining sample was further diluted 5 times in PBS and analyzed on the Cyflow for indirect bead-based measurements of CD4 cells. During acquisition, the trigger was set on FL3 (CD3), with the threshold set high enough to exclude RBCs and unstained white blood cells (WBCs). Absolute CD4 counts were derived from the FL1 × FL2 dot plot analysis as well as the ratio of the gated $CD3^+CD4^+$ cells and corresponding reference beads with known concentration (provided by the manufacturer). This type of analysis obviously requires an instrument equipped with at least 2 fluorescence detectors.

For the TruCount analysis on the FACScan, 50 μL of whole blood was pipetted into polystyrene TruCount tubes. Ten microliters of a monoclonal antibody cocktail containing CD3-FITC, CD4-PE, and CD45-PerCP was added to the blood in the test tubes and left to incubate for 15 minutes at room temperature. Before sample acquisition on the FACScan or on Cyflow, RBCs were lysed by adding 2 mL of RBC lysing solution (Becton Dickinson). The absolute CD4 cell counts were derived from the known bead concentration (provided by the manufacturer) in the TruCount tubes.

Statistical Analysis

Correlations between the absolute $CD4^+$ counts obtained by the different methods were analyzed by the Passing and Bablok method,[9,10] which, in common with all nonparametric methods, is less sensitive to outliers. This method provides a test of the agreement of 2 analytic methods. Difference plots are given as proposed by Bland and Altman.[11] The Bland-Altman method examines, in a discriminative fashion, whether the methods agree sufficiently well to be used interchangeably. The average of the 2 results from the 2 methods is displayed on X and plotted against the difference between the 2 methods shown on Y.

The average difference between the 2 methods, referred to as "bias," is marked on the graph by a horizontal line, and the mean difference and limits of agreement with a 95% confidence interval (CI) were also calculated. The level of significance for linear regression was set at $\alpha < 0.05$. "Method Validator" version 1.1.9.0 software was used to perform the statistical analyses.

RESULTS

Parallel CD4 measurements on all the instruments were available on 121 of 130 blood samples tested. CD4 counting was automatically aborted by the FACSCount during sample acquisition of 9 samples, which were excluded from further analysis. Table 1 gives a detailed overview of the average and median CD4 counts, the standard deviation, and the minimum and maximum CD4 counts obtained for 121 samples according to the instrument or CD4 procedure used. The average CD4 count for 121 blood samples tested on the FACSCount, the predicate CD4 counting instrument in this study, was 423 CD4 cells/μL, and results ranged from 2 to 1808 cells/μL (median = 345 cells/μL). Table 2 provides an overview of the agreement between the various methods.

TABLE 1. Overview of Absolute CD4 Measurements on Single-Platform Flow Cytometers: Cyflow, FACSCount, and TruCount on FACScan Using Direct Volumetric and Bead-Based CD4 Measurements

Technology	RBC Lysis	*n*	Average CD4 Count	SD	Median	Minimum	Maximum
Volumetric							
Cyflow	NLNW	121	418	380	302	1	1996
Cyflow	LNW	121	406	361	305	1	1907
Bead-based							
FACScan TruCount	LNW	121	410	351	317	2	2059
Cyflow TruCount	LNW	121	396	338	313	5	1972
FACSCount	NLNW	121	423	336	345	2	1808
Cyflow FACSCount	NLNW	121	429	347	349	4	1940

Data represent CD4 counts per microliter. The range of 121 CD4 measurements is given as the minimum and maximum. Cyflow TruCount represents the analysis of TruCount tubes on the Cyflow. Cyflow FACScount represents the analysis of FACScount tubes on the Cyflow.

Evaluation of Direct Volumetric CD4 Measurements on the Cyflow Using Unlysed and Lysed Whole Blood

Figure 1A and B compare volumetric CD4 measurements in unlysed whole blood (NLNW) on the Cyflow SL blue with CD4 measurements on the FACSCount (NLNW but bead-based). The volumetric CD4 measurements on the Cyflow agree well with the CD4 results of the FACSCount ($R^2 = 0.97$, mean difference = −4 cells/μL, 95% CI: −18–10). The median CD4 count, however, was higher on the FACSCount (345 cells/μL) than on the Cyflow (302 cells/μL).

Direct volumetric CD4 measurements in lysed whole blood (LNW) were performed on the Cyflow using the panleucogating procedure. The Cyflow CD4 results were compared with those obtained by TruCount analysis (LNW but bead-based) on the FACScan. As shown in Figure 1C and D, the volumetric LNW CD4 results on the Cyflow were comparable to the bead-based CD4 results on FACScan using TruCount ($R^2 = 0.97$, mean difference = −4, 95% CI: −14–7). Similarly, the median CD4 count was slightly higher on the FACScan (317 cells/μL) than on the Cyflow (305 cells/μL).

To study the effect of RBC lysis on the volumetric CD4 measurements, we compared volumetric CD4 measurements in unlysed (NLNW) and lysed (LNW) whole blood on the Cyflow. As shown in Figure 2C and D, the LNW method resulted in a slightly lower average CD4 count (406 cells/μL) than the NLNW method (418 cells/μL) ($R^2 = 0.98$, mean difference = −12, 95% CI: −21 to −3). The median CD4 counts, however, were comparable (305 cells/μL vs. 302 cells/μL, respectively).

The effect of RBC lysis could also be assessed by comparing TruCount (LNW) and FACSCount (NLNW) results. As shown in Figure 2A, the average CD4 count by TruCount (410 cells/μL) was slightly lower than that by FACSCount (423 cells/μL) ($R^2 = 0.98$, mean difference = −12, 95% CI: −23 to −1). The median CD4 count, however, was markedly (8%) lower on the FACScan using TruCount (317 cells/μL) than that by FACSCount (345 cells/μL).

TABLE 2. Bland-Altman Analysis Comparing Flow-Based Technologies for Absolute CD4 Counting Using Single-Platform Bead-Based and Direct Volumetric Measurements

CD4 Counting Method	Mean Difference	95% CI	Mean Range	Limits of Agreement
Cyflow NLNW vs. FACSCount	−4	−18–10	1–1902	−161–−153
Cyflow LNW vs. TruCount	−4	−14–7	1–1983	−121–−114
TruCount vs. FACSCount	−12	−23–−1	2–1933	−134–109
Cyflow LNW vs. NLNW	−12	−21–−3	1–1951	−111–87
FACSCount on Cyflow vs. FACSCount	6	2–11	3–1874	−47–60
TruCount on Cyflow vs. FACScan	−14	−20–−9	3–2015	−73–45

Data (n = 121) are expressed as CD4 cells/μL. Mean difference is the difference of the mean CD4 values of 2 CD4 counting methods. 95% CI is the CI of the mean difference. Mean range is the range of the average CD4 count of 2 CD4 counting methods. Limits of agreement indicate the 95% CI of the differences between 2 CD4 counting methods.

Evaluation of Indirect Bead-Based Absolute CD4 Measurements on the Cyflow Using Unlysed and Lysed Whole Blood

To assess the performance of bead-based, and thus indirect absolute CD4 measurements, in unlysed (NLNW) whole blood on the Cyflow, we analyzed FACSCount reagents on the FACSCount and Cyflow. The CD4 concentration on the Cyflow was calculated from the bead concentration provided by the FACSCount manufacturer. Figure 3A and B show that the agreement between the bead-based CD4 measurements on both systems was excellent ($R^2 = 0.99$, mean difference = 6, 95% CI: 2–11).

To assess bead-based indirect absolute CD4 measurements in lysed whole blood (LNW) on the Cyflow, we analyzed TruCount tubes on the Cyflow and FACScan. Again, excellent correlations were found between both systems using the TruCount reagents (see Fig. 3C and D; $R^2 = 0.99$, mean difference = −14 cells/μL, 95% CI: −20 to −9). The median CD4 counts were similar on the FACScan (317 cells/μL) and the Cyflow (313 cells/μL).

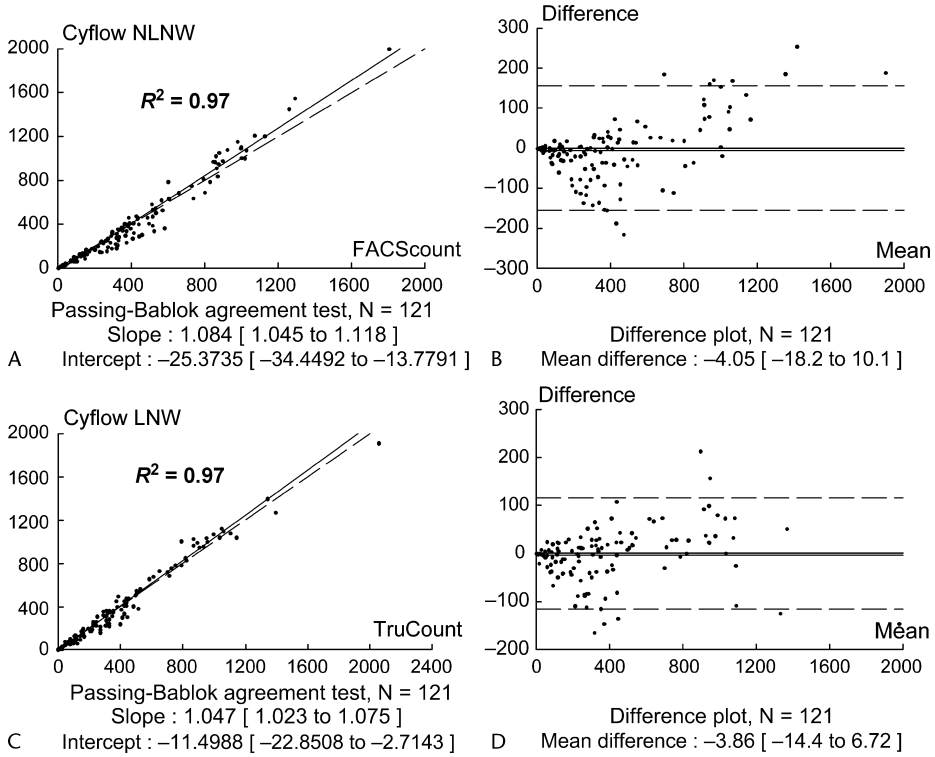

FIGURE 1. A, Passing-Bablok agreement test between volumetric CD4 measurements on the Cyflow and CD4 cell counting on the FACScount. The latter was used as the predicate instrument for CD4 measurements in unlysed whole blood (no-lyse-no-wash [NLNW]) on the Cyflow. B, Bland-Altman analysis of the comparison in A. C, Passing-Bablok agreement test between volumetric CD4 counting on the Cyflow and bead-based (TruCount) CD4 counting on the FACScan. The latter was used as the predicate instrument for CD4 measurements in lysed whole blood (lyse-no-wash [LNW]) on the Cyflow. D, Bland-Altman analyses of the comparison in C.

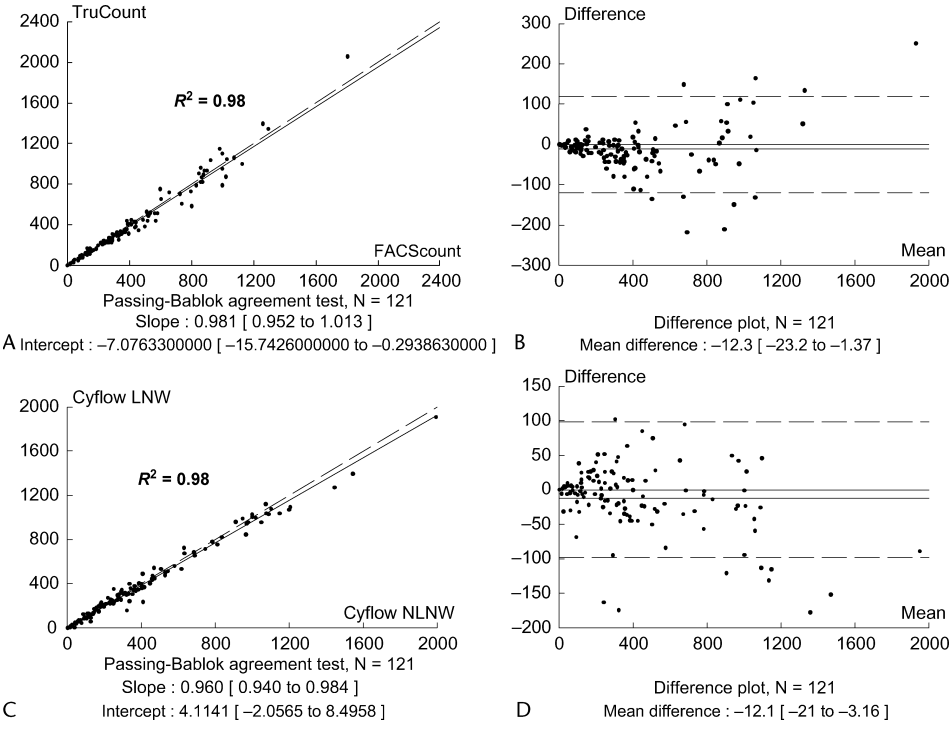

FIGURE 2. A, Passing-Bablok agreement test comparing absolute CD4 counting in lysed (lyse-no-wash [LNW]) and unlysed (no-lyse-no-wash [NLNW]) whole blood on 2 predicate CD4 counting instruments: TruCount on the FACScan (LNW) and FACScount (NLNW). B, Bland-Altman analysis of the comparison in A. C, Passing-Bablok agreement test comparing direct volumetric CD4 counting in lysed (LNW) and unlysed (NLNW) blood on the Cyflow. D, Bland-Altman analysis of the comparison in C.

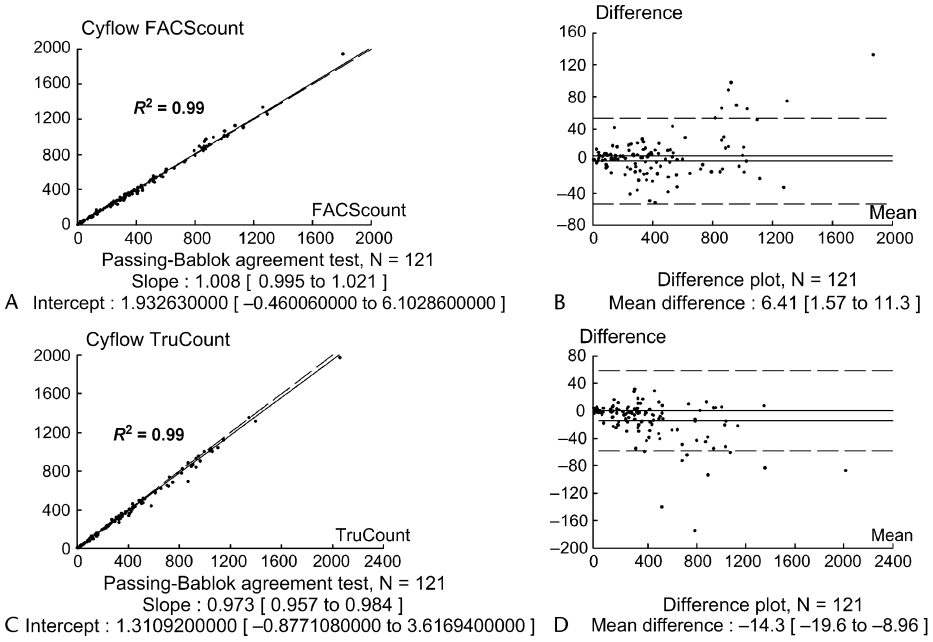

FIGURE 3. A, Passing-Bablok agreement test comparing 2 bead-based absolute CD4 measurements in unlysed whole blood (no-lyse-no-wash [NLNW]). FACScount tubes (NLNW) were analyzed on the Cyflow and FACScount. B, Bland-Altman analysis of the comparison in A. C, Passing-Bablok agreement test comparing 2 bead-based absolute CD4 measurements in lysed whole blood (lyse-no-wash [LNW]). TruCount tubes (LNW) were analyzed on the Cyflow and FACScan. D, Bland-Altman analysis of the comparisons in C.

Evaluation of the Stability of Volumetric Analyses on the Cyflow by Monitoring Recovery of Reference Beads in TruCount and FACScount Tubes Analyzed on the Cyflow

The direct volumetric measurements on the Cyflow allowed us to monitor the total bead recovery in a fixed volume as an internal quality control during the bead-based CD4 measurements on the Cyflow. Figure 4 illustrates the relative bead recovery as percent of the expected bead count in 121 samples during analysis of the FACSCount and TruCount tubes on the Cyflow. On average, 91% ± SD of 15% of the FACSCount beads and 94% ± SD of 12% of the TruCount beads could be recovered on the Cyflow. This decreased bead recovery was not systematic but occurred at different time intervals, particularly during the acquisition of the first 60 samples, where the bead recovery dropped to 80% of the expected value.

DISCUSSION

Since the introduction of antiretroviral therapy (ART) in developing countries, the need for more affordable and/or alternative absolute CD4 counting technologies has increased significantly. Several alternative manual CD4 tests have been introduced in the past decade, but they are not nearly as precise as clinical flow cytometers and have serious limitations with regard to sample throughput and workload.[3,12] We evaluated the accuracy of a mobile, single-platform, volumetric, multiparameter flow cytometer (Cyflow SL blue) for absolute CD4 T-cell counting in HIV patients in Dakar, Senegal. Our results indicate that unlysed and lysed whole blood could be used to obtain relatively accurate absolute CD4 measurements on this Cyflow instrument. Taking into consideration the relatively high cost of multiparametric CD4 T-cell assays performed on a single platform by using microbead-based technology, low-cost volumetric flow cytometers like the Cyflow may offer an attractive affordable alternative, particularly in resource-poor settings.

Volumetric measurements on the Cyflow are based on automatic detection of the sample fluid level by 2 electrodes that trigger start and stop of sample acquisition. Incorrect reading of the sample fluid level, caused by the presence of air bubbles in sample tubes during acquisition, for example, has a negative effect on accuracy, and technicians should be trained to avoid this kind of error. Therefore, we tested 2 bead-based absolute CD4 methods that do not depend on precise volume detection, the first in lysed blood and the second in unlysed blood, as a putative internal quality control tool for direct volumetric CD4 measurements on the Cyflow. For routine volumetric CD4 measurements, reference beads are not required on the Cyflow and are too expensive, but their regular use was considered important to ascertain reliable volumetric

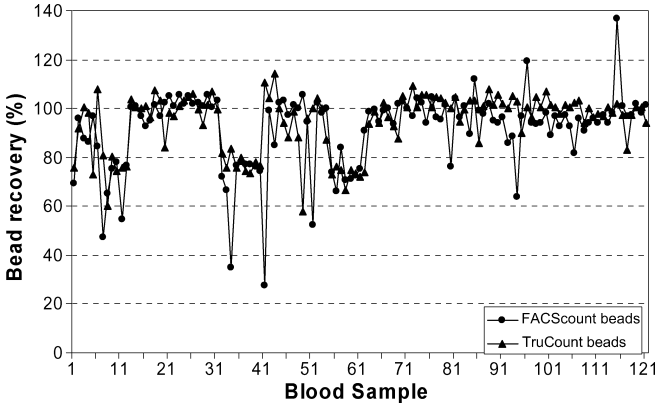

FIGURE 4. The precision of the volumetric CD4 measurements on the Cyflow during this study was assessed by measuring the relative (%) recovery of reference beads in the FACScount and TruCount tubes that were analyzed on the Cyflow.

CD4 measurements over time. We have shown that TruCount and FACScount reagents or their equivalent may be adequate internal quality control tools for CD4 measurements in lysed or unlysed whole blood on the Cyflow. They could be an important supplement to external quality control programs, which are often lacking in resource-poor countries

The accuracy of the volumetric measurements on the Cyflow was also assessed by monitoring the relative bead recovery in bead-based CD4 measurements. We noticed several times that for some reason, the relative recovery of reference beads in a fixed volume dropped to less than 80% of the expected bead count. This occurred 3 times for a consecutive set of blood samples and was independent of the staining protocol (RBC lysis or not) and type of beads used (TruCount or FACScount beads). It may also indicate that a user's experience with the instrument is crucial to ensure reliable results. The instrument under evaluation had a built-in time parameter that allowed monitoring of the cell acquisition rate and stability of fluorescence intensity during sample acquisition. This feature was a useful internal quality control instrument during the study.

Comparison of absolute CD4 measurements in lysed and unlysed whole blood allowed us to study the effect of RBC lysis on the accuracy of absolute CD4 measurements. We found that absolute CD4 counts were only slightly lower in lysed blood than in unlysed blood and the effect of RBC lysis was considered negligible. It certainly was less impressive than other studies have suggested.[13]

We performed CD4 counting on a 5-parameter Cyflow instrument using a mixture of 3 antibodies: the first to identify WBCs (CD45), the second to identify T cells (CD3), and the third to identify CD4 T cells (CD4). Primary CD4 gating (using only anti-CD4 antibody) in whole blood has been proposed as a logical next step to reduce the cost of a CD4 cell count.[14] Since the evaluation of this particular instrument, a new version of the Cyflow, the Cyflow Counter, equipped with a green laser, only 1 fluorescence detector, and no scatter detectors, has been released. It is obvious that CD4 counting procedures that are based on multiple-parameter analyses such as panleucogating are not applicable on the Cyflow Counter. Single-parameter CD4 gating using a single monoclonal antibody is indisputably more affordable than any multiparameter CD4 measurement, but accuracy and precision may be seriously affected as a result of the interference of monocytes, particularly in samples with low CD4 counts ($<$200 cells/μL). Monocytes express CD4 molecules at their cell surface, and they are not always well separated from $CD4^+$ T cells in single-parameter CD4 analyses by flow cytometry. Nevertheless, single-parameter flow cytometry instruments like the Cyflow Counter and even the more complicated dual-parameter systems like the Cyflow SL green may easily out-compete any currently available manual CD4 counting method in terms of accuracy, precision, and cost per test, and, certainly, in sample throughput.[15] Data from a recent multicenter evaluation of different Cyflow instrument types in the field look promising,[16] but other multicenter field studies independent of the manufacturer are urgently needed to validate the robustness of CD4 counting by single-parameter flow cytometry in clinical settings in developing countries. In addition, such field evaluation should address issues like instrument down time, troubleshooting, and service and repair efficiency in the field, because these are at least as important as the instrument's accuracy and precision performance.

REFERENCES

1. Egger M, May M, Chene G, et al. Prognosis of HIV-1-infected patients starting highly active antiretroviral therapy: a collaborative analysis of prospective studies. *Lancet*. 2002;360:119–129.
2. Janossy G, Jani IV, Kahan M, et al. Precise CD4 T-cell counting using red diode laser excitation: for richer, for poorer. *Cytometry*. 2002;50:78–85.
3. Crowe S, Turnbull S, Oelrichs R, et al. Monitoring of human immunodeficiency virus infection in resource-constrained countries. *Clin Infec Dis*. 2003;37(Suppl 1):S25.
4. Koepke JA, Landay AL. Precision and accuracy of absolute lymphocyte counts. *Clin Immunol Immunopathol*. 1989;52:19–27.
5. Brando B, Barnett D, Janossy G, et al. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. European Working Group on Clinical Cell Analysis. *Cytometry*. 2000;42:327–346.
6. Strauss K, Hannet I, Engels S, et al. Performance evaluation of the FACSCount system: a dedicated system for clinical cellular analysis. *Cytometry*. 1996;26:52–59.
7. Nicholson JKA, Hubbard M, Jones B. Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. *Cytometry*. 1996;26:16–21.
8. Glencross D, Scott LE, Jani IV, et al. CD45-assisted panleucogating for accurate, cost-effective dual-platform CD4+ T-cell enumeration. *Cytometry*. 2002;50:69–77.
9. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. *J Clin Chem Clin Biochem*. 1983;21:709–720.
10. Passing H, Bablok W. Comparison of several regression procedures for method comparison studies and determination of sample size. *J Clin Chem Clin Biochem*. 1984;22:431–445.
11. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*. 1986;1:307–310.
12. Didier JM, Kazatchkine M. Comparative assessment of five alternative methods for CD4 T-lymphocytes enumeration for implementation in developing countries. *J Acquir Immune Defic Syndr*. 2001;26:193–195.
13. Göhde W, Cassens U, Lehman LG, et al. Individual patient-dependent influence of erythrocyte lysing procedures on flow-cytometric analysis of leukocyte subpopulations. *Transfus Med Hemother*. 2003;30:165–170.
14. Janossy G, Jani I, Gohde W. Affordable CD4+ T-cell counts on "single platform" flow cytometers I. Primary CD4 gating. *Br J Haematol*. 2000; 111:1198–1208.
15. Imade GE, Badung B, Pam S, et al. Comparison of a new, affordable flow cytometric method and the manual magnetic bead technique for CD4 T-lymphocyte counting in a northern Nigerian setting. *Clin Diagn Lab Immunol*. 2005;12:224–227.
16. Cassens U, Gohde W, Kuling G, et al. Simplified volumetric flow cytometry allows feasible and accurate determination of CD4 T lymphocytes in immunodeficient patients worldwide. *Antivir Ther*. 2004;9: 395–405.