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Cost Savings by Reagent Reduction in Flow Cytometry-Based CD4+ T Cell Counts: An Approach to Improve Accessibility for HIV Management

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SUMMARY In Thailand, the cost of antiretrovirals has recently been reduced more than 10 fold. Likewise strategies for a cost reduction in laboratory monitoring are warranted. This study was designed to explore if the most expensive reagent in flow cytometry based CD4+ cell monitoring, the $CD4^+$/$CD8^+$ monoclonal antibodies, can be reduced without a loss of accuracy. Blood samples from 55 HIV seronegative (HIV-) and 76 HIV+ subjects were analyzed for %$CD4^+$ and %$CD8^+$ T cells using a two color monoclonal antibody panel (BD Biosciences, CA, USA) with 3 different amounts of the recommended reagents for staining: 1) standard, 2) half, and 3) one-fourth. A significant Spearman correlation of 0.987 was shown for the %$CD4^+$ T cell test results for one half as well as one-fourth of the recommended amount compared to the standard staining according to the manufacturer's instruction ($p < 0.0001$). For the %$CD8^+$ T cell test results, the correlation between the standard and the half or one-fourth reduced staining was 0.972 ($p < 0.0001$). Bland-Altman analysis showed no significant bias between the results from one half or one-fourth of the recommended amount *versus* the standard. The sensitivity and specificity of the two methods at the $CD4^+$ T cell count cut-off of 200 cells/μl were 93% and 100%; and 96% and 99%, respectively. Our study indicates that a reduction of the reagents to half or one-fourth of the amount recommended by the manufacturer was still able to generate reliable results for $CD4^+$ and $CD8^+$ T cell counts. Such an approach will significantly reduce the cost of $CD4^+$ monitoring for resource limited settings where a flow cytometer is available.

$CD4^+$ T lymphocytes are the main targets of the human immunodeficiency virus (HIV). HIV-infected T cells are destroyed by various mechanisms such as virus-induced cytolysis, CD8-mediated cytolysis and syncytial formation.[1-4] The infection will eventually result in a progressive decline in the number and function of $CD4^+$ T cells. The absolute number of $CD4^+$ T cells determines whether a patient is susceptible to a particular opportunistic pathogen. For instance, *Pneumocystis carinii* pneumonia has a high chance to occur when the $CD4^+$ T cell count of a patient drops below 200 cells/μl.[5,6] Similarly, CMV retinitis and *Mycobacterium avium* complex (MAC) infections tend to occur in patients with $CD4^+$ T cell counts below 100 cells/μl.[1,2,7-9] Therefore, the number of $CD4^+$ T cells is a good prognostic marker for patients with HIV infection. Appropriate chemopro-

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phylaxis can be initiated when a critical level of $CD4^+$ T cells is reached.[10] Antiretroviral therapy (ART) is currently the mainstay in the management of HIV-infected patients. It has been shown to improve quality of life and even prolong the life of HIV-infected patients.[11,12] The clinical benefit is due to a durable HIV viral suppression and an increase in the number of $CD4^+$ T cells following ART. Current HIV management guidelines are recommending commencing triple combination antiretroviral therapy when $CD4^+$ T cell counts fall below 350 cells/μl.[13-15] Most of the developing countries including Thailand, however, recommend a $CD4^+$ T count of 200 cells/μl as the cut-off for commencing antiretroviral therapy. Thus, the $CD4^+$ T cell count, ideally in conjunction with plasma HIV-1 RNA, is essential in monitoring the patients prior to/and during antiretroviral therapy.[16-18] In Thailand, the cost of antiretrovirals has recently been reduced more than 10 fold. Similarly, research for reducing the cost of $CD4^+$ T cell counts and of viral load monitoring is warranted for countries with limited resources.

The standard methodology for determining absolute $CD4^+$ T cell counts is an immunophenotypic identification of cells with fluorescence-labeled monoclonal antibodies directed against the $CD4^+$ antigen.[19] Relative percentages of $CD4^+$ T cells are determined with a flow cytometer. An absolute $CD4^+$ T cell count is derived by multiplying the percentage of $CD3^+CD4^+$lymphocytes with the absolute lymphocyte count determined by hematology instruments. Thus, in this study we evaluated whether a reduction of the $CD4^+$ and $CD8^+$ staining reagents from the standard volume recommended by the manufacturer is feasible. We have validated reagent volume reductions to either one half or one-fourth by determining $CD4^+$ and $CD8^+$ percentages using a flow cytometer for blood samples of both HIV seronegative and HIV-infected individuals.

MATERIALS AND METHODS

Sample collection

Peripheral blood samples from 55 HIV-seronegative blood donors of the Thai Red Cross National Blood Center and from 76 HIV-seropositive individuals who attended the HIV clinic at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, were collected into potassium ethylenediamine tetraacetic acid (K_3EDTA) vacutainer tubes (3 ml per tube), two for each individual. One sample was sent to the central laboratory of Chulalongkorn Hospital for a complete blood count (CBC), and the other sample was used for the $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cell determinations.

Flow cytometric immunophenotyping for $CD4^+$ and $CD8^+$ T lymphocytes

The monoclonal antibody panels used for labeling the specimens are listed in Table 1. Simultest (BD Biosciences) two-color antibody panels were used for cell staining and SimulSET software (BD Biosciences) was used for automated data collection. Each blood sample was simultaneously stained in three different settings as described in Table 2. The "standard" setting was the method recommended by the manufacturer of the monoclonal antibodies. The "half" setting was carried out by reducing the volume of the recommended monoclonal antibody by half and the blood volume from 100 to 50 μl. The working FACS lysing solution was also reduced from 2 to 1 ml. The "one-fourth" setting was carried out by using only ¼ of the standard volume of monoclonal antibody. The volumes of blood and of the working FACS lysing solution were only reduced

Table 1 Two color monoclonal antibody panel (BD Biosciences, CA, USA)

Tube no.	FITC	PE	Cell population identified
1	CD45	CD14	Lymphocytes, monocytes and granulocytes
2	CD3	CD4	$CD4^+$ T cells
3	CD3	CD8	$CD8^+$ T cells

FITC, fluorescein isothiocyanate
PE, phycoerythrin.

by half as in the "half" setting. The 1994 Centers for Disease Control Revised Guideline[20] for the performance of CD4 determinations was followed in regards to sample processing during two-color staining, washing and fixing. In brief, EDTA-anticoagulated blood was incubated with the designated antibodies for 20 minutes at 25°C and the erythrocytes were lysed by FACS lysing solution. The centrifuged cells were washed by PBS, and then fixed by 1% paraformaldehyde. The stained cells were analyzed on the same day by SimulSET software.

Analyses

The criteria for accepting data with the SimulSET software included the following: (i) gated lymphocyte purity > 85 %, (ii) lymphocyte recovery within the gate > 90%, and (iii) differences in the $CD3^+$ percentages between the $CD3^+CD4^+$ and $CD3^+CD8^+$ tube of < 7%. The % $CD4^+$ and % $CD8^+$ test results were analyzed for a correlation of their performances by Spearman Correlation (GraphPad Prism® version 3.0, GraphPad Software, San Diego California USA) and by Bland-Altman (MedCalc version 9.2).

RESULTS

The median percentages and median absolute $CD4^+$ and $CD8^+$ T cell counts of the three different staining methods are shown in Table 3. Scatter plots of the correlations of the percent $CD4^+$ T cell results between standard staining and half-reagent staining as well as between standard staining and one-fourth reagent staining are shown in Fig. 1A and 1B, respectively. Similar plots of the correlations of the percent $CD8^+$ T cell results are shown in Fig. 2A and 2B. The correlations of percent $CD4^+$ and percent $CD8^+$ T cells of standard staining *versus* half reagent staining, and of standard staining *versus* one-fourth reagent staining of all subjects were 0.987, 0.986 and 0.972, 0.972, respectively (Spearman *r*, $p < 0.0001$).

To uncover a possible bias between the methods, Bland-Altman analyses were performed. As illustrated in Figs. 3 and 4, the Bland-Altman analyses showed that both percent $CD4^+$ and percent $CD8^+$ T cells stained by using half or one-fourth of the reagents agree sufficiently within mean ± 2SD and can be used interchangeably. The percent $CD4^+$

Table 2 Sample and reagent volumes used for staining

Sample and reagent volume	Experimental setting		
	Standard*	Half**	One-fourth***
Whole blood (μl)	100	50	50
Monoclonal antibody (μl)	20	10	5
FACS lysing solution (ml)	2	1	1

*Monoclonal antibody as per the manufacturer's instruction (20 μl per sample)
**Monoclonal antibody reduced to one half of the recommended volume (10 μl per sample)
***Monoclonal antibody reduced to one-fourth of the recommended volume (5 μl per sample)

Table 3 Summary of the comparative results of three different staining assays

	Overall subjects					HIV-seronegative subjects					HIV-seropositive subjects				
		Median					Median					Median			
	N	CD4		CD8		N	CD4		CD8		N	CD4		CD8	
		%	Abs	%	Abs		%	Abs	%	Abs		%	Abs	%	Abs
Standard	131	26	484	43	773	55	39	922	29	736	76	16	286	56	853
Half	131	26	470	43	760	55	40	918	29	717	76	16	280	55.5	854
One-fourth	131	25	457	42	748	55	40	926	28	712	76	16	246	52.5	789

T cell counts estimated by the half and one-fourth reagent volumes were slightly higher (bias, +0.2824; 95% confidence interval (CI) -0.0909 to +0.6558, limit of agreement between +4.5 and -4.0) and slightly lower (bias, -0.6031; 95% CI, -1.0028 to -0.2033, limit of agreement between +3.9 and -5.1) as compared to the uses of the manufacturer recommended standard volume of reagent, respectively Slightly lower percentage of $CD8^+$ T cell counts were observed when half- or one-fourth reagent volumes were used (bias, -0.3664; 95% CI,-0.9477 to +0.2149, limit of agreement between +6.2 and -7.0 and bias, -1.3130; 95% CI, -2.1141 to -0.5119, limit of agreement between +7.8 and -10.4, respectively).

Sensitivity and specificity were calculated based on the $CD4^+$ T cell cut-off at 200 cells/μl. The half reagent reduction method showed 93% sensitivity and 100% specificity, whereas the one-fourth reagent reduction method showed 96% and 99%, respectively (Table 4).

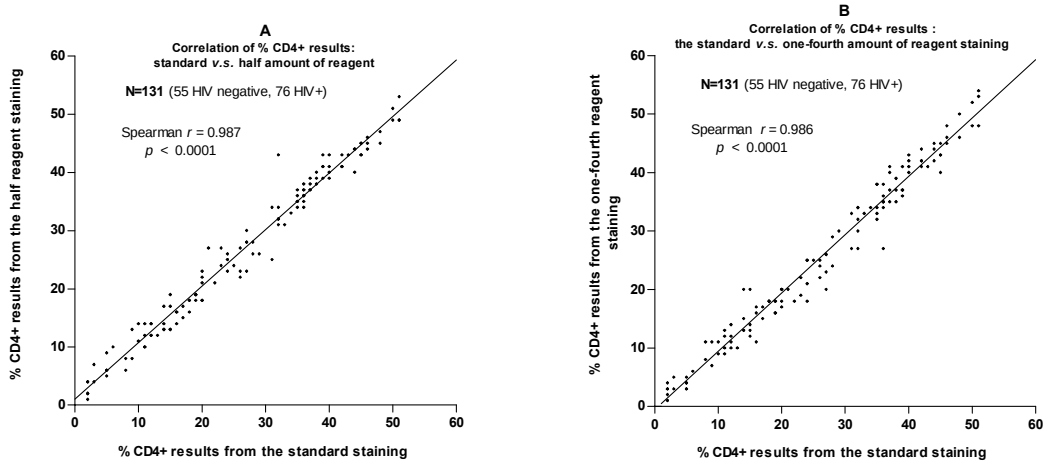

Fig. 1 Correlation plots of %$CD4^+$ T cell count results between standard staining versus half-reagent staining (A), and between standard staining versus one-fourth reagent staining (B).

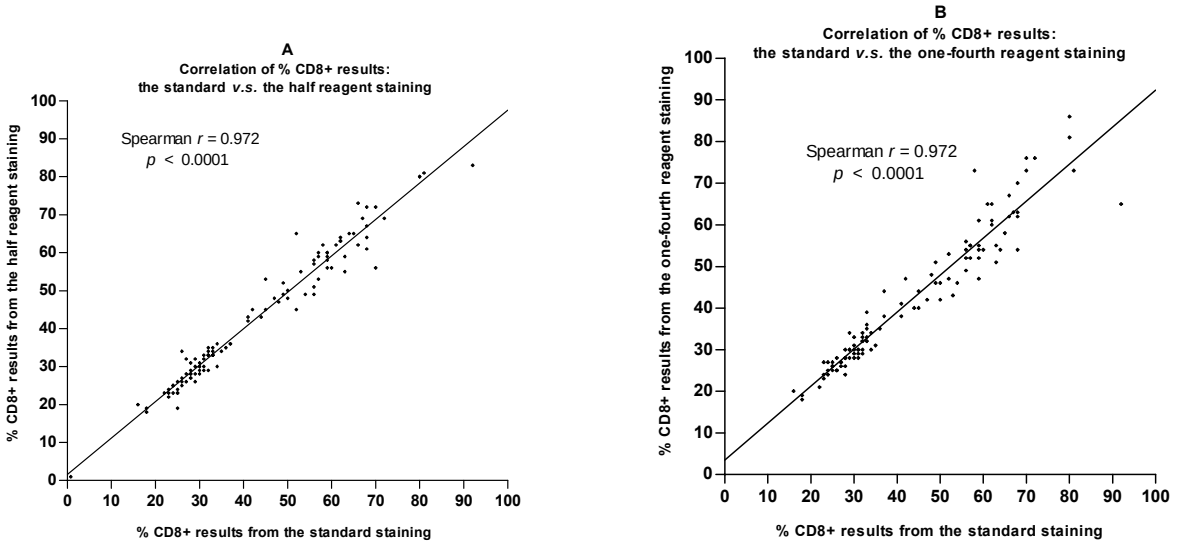

Fig. 2 Correlation of %$CD8^+$ T cell count results between standard staining versus half-reagent staining (A), and between standard staining versus one-fourth reagent staining (B).

DISCUSSION

We have explored 2 approaches to reduce the cost of the $CD4^+$ T cell counts namely the "half" and "one-fourth" reagent methods. The half reagent approach was a proportional scaling down to half the recommended volumes of monoclonal antibody (from 20 to 10 µl), blood sample (from 100 to 50 µl), and FACS lysing solution (from 2 to 1 ml). The second approach of this study reduced the amount of the staining monoclonal antibody to one-fourth of the recommended volume. The actual volume of the monoclonal antibody used in the "one-fourth" setting was 5 µl which can still be accurately delivered. However, the blood sample volume was half of the standard protocol, *i.e*. 50 µl, as a further reduction to one-fourth of the standard blood sample volume, *i.e.* 25 µl, might compromise the accuracy particularly in HIV-infected patients with leucopenia and low $CD4^+$ T cell counts.

The results of the $\%CD4^+$ and $\%CD8^+$ T cells generated both by the half and one-fourth reagent methods correlated significantly with the recommended standard reagent results as shown by Spearman correlations of 0.99 and 0.97, respectively ($p < 0.0001$) (see Figs. 1 and 2). Bland-Altman analysis showed no significant bias towards these two reagent reduction methods as evidenced by less than 2% of the results deviating greater than the mean ± 2SD (see Figs. 3 and 4). In terms of clinical application of these cost-reduction approaches, at a 200 cells/µl cut-off of the $CD4^+$ T cell count, the sensitivity and specificity of the half and one-fourth reagent methods were 93%, 100%; and 96%, 99%, respectively. Thus, all analysis results indicated that the monoclonal antibodies and the FACS lysing solution could be reduced to half or even to one-fourth while providing reliable results compared to the volumes recommended by the manufacturer.

Table 4 Sensitivity and specificity results of the half reagent and one-fourth reagent stainings at a 200 cells/µl cut-off of the $CD4^+$ T cell counts. The manufacturer's standard reagent staining was used as a gold standard

Setting	Standard reagent staining			
	Sensitivity	Specificity	Absolute $CD4^+$ T cells of subject < 200 cells/µl	Absolute $CD4^+$ T cells of subject ≥ 200 cells/µl
Half	93	100	27	104
One-fourth	96	99	27	104

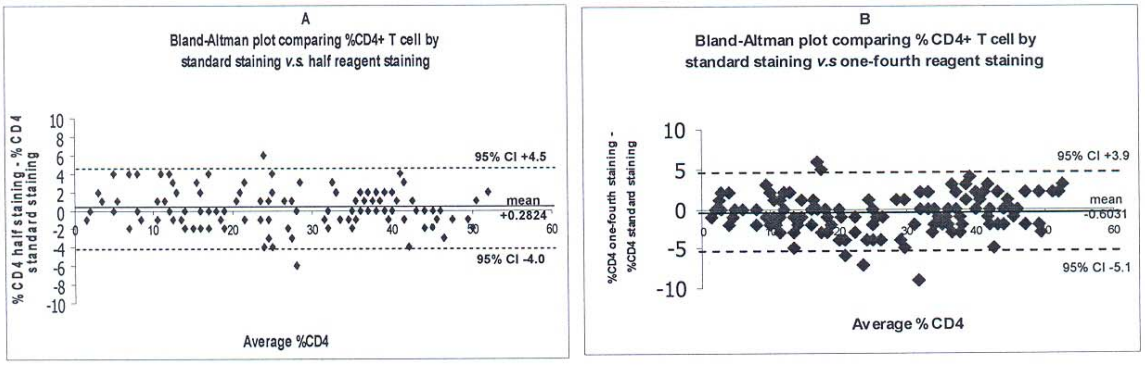

Fig. 3 Bland-Altman plot comparing $\%CD4^+$ T cell count by standard staining versus half reagent staining (A), and between standard staining versus one-fourth reagent staining (B). The dark continuous line drawn indicates the bias (mean difference), and the dotted lines are the limits of agreement (95% CI).

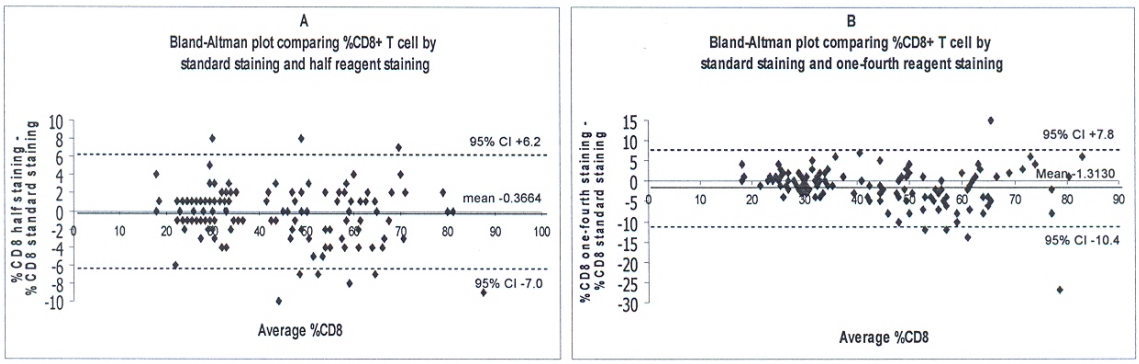

Fig. 4 Bland-Altman plot comparing %CD8$^+$ T cell count by standard staining versus half reagent staining (4A), and between standard staining versus one-fourth reagent staining (4B). The dark continuous line drawn indicates the bias (mean difference), and the dotted lines are the limits of agreement (95% CI).

In our study, blood sample volumes of 50 µl were used. It needs to be emphasized that the median count in our patient population was 286 cells/µl (range 10-1733), and 27 out of 131 subjects had a CD4$^+$ T cell count < 200 cells/µl. The reliability of the proportionate reduction of the blood sample volume by half therefore needs to be evaluated in a larger sample size of patients with CD4$^+$ T cell counts < 200 cells/µl.

This is the first report on an attempt to reduce the cost of CD4$^+$/CD8$^+$ T cell staining by reducing the amounts of the staining reagents. However, each laboratory needs to perform its own evaluation before using this method, particularly with a monoclonal antibody from other commercial sources than ours. The one-fourth reagent staining method with monoclonal antibodies from BD Biosciences is the technique routinely used in clinical trials and clinical service in our center. The validity of our CD4$^+$ T cell results has been certified by various international monitoring and auditing agencies such as QASI of The Flinders University of South Australia, QASI of Clinical Pathology Accreditation (UK) Ltd., QASI of Health Canada and IQAP of Beckman Coulter, Inc. Thus this cost-saving CD4$^+$/CD8$^+$ T cell staining technique should be considered in countries with restricted resources in evaluating their HIV-infected patients. However, in extreme resource constraint countries where flow cytometry is not available, other non-flow cytometric CD4$^+$ T cell measurement techniques may be more appropriate.[21-23] Nevertheless, most of these investigational systems are labor intensive and difficult to scale up in order to match the expanding access to antiretrovirals. To improve the non-flow cytometry CD4$^+$ T cell counts the labor intensive steps need to be removed, thus a non-flow cytometic method based on automated Dynabeads® CD4$^+$ T cell counts is currently being developed and evaluated in our laboratory. Of note, recently a low cost generic monoclonal anti-CD4$^+$ reagent has become available in Thailand;[24] this will also facilitate the access to CD4$^+$ T cell count monitoring for patients under antiretroviral therapy.

In conclusion, two color monoclonal antibody panels (BD Biosciences, CA, USA) reduced to one half or one-fourth of the recommended amounts together with a reduced blood sample size of 50 µl can be used interchangeably with the recommended volumes as per the manufacturer's instructions. This reagent reduction approach reduces the cost of CD4$^+$ T cell counts by 50-75%. Thus it makes CD4$^+$ T cell monitoring more accessible for HIV-infected patients. More efforts are, however, required to bring both antiviral therapy and proper laboratory monitoring to HIV-infected individuals in resource constrained settings.

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