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Evaluation of automated flow cytometer single-platform for absolute CD4⁺ T-lymphocytes enumeration in HIV patients, Trat Province, Thailand

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ABSTRACT

Background: The routine analysis of CD4⁺ T-lymphocyte percentages and absolute counts in HIV patients commonly employs the dual-platform method (blood cell analyzer and flow cytometer). However, variability related to equipment, methodology, or operator performance may affect accuracy. This study introduces the single-platform method, which relies solely on a flow cytometer to reduce variability, streamline workflow, and shorten turnaround times, in alignment with the Rational Laboratory Use guidelines of the Department of Medical Sciences, Ministry of Public Health.

Objectives: To evaluate and compare the single-platform and dual-platform methods for determining CD4⁺ T-lymphocyte percentages and absolute counts.

Materials and methods: The study was conducted from January 24 to November 29, 2024, encompassing the entire research process — from conceptual development, problem analysis, and study design to data collection, statistical analysis, interpretation, and application of findings. Routine diagnostic data were collected from HIV-infected patients in Trat Province, Thailand, between April 1 and July 31, 2024, as part of the data collection phase. Samples were analyzed using a semi-automated blood cell analyzer and a flow cytometer, and results were compared with those obtained using the single-platform method, which employed only the flow cytometer. Statistical analyses were performed to assess the accuracy, precision, and reliability of the single-platform method compared with the conventional approach. Descriptive statistics, correlation, linear regression, and Bland–Altman analysis were applied to evaluate agreement and systematic bias. All statistical tests were conducted at a 95% confidence interval, with *p*-values <0.05 considered statistically significant.

Results: Both methods produced data that followed a normal distribution (Kolmogorov–Smirnov test; $p>0.05$). Correlation coefficients demonstrated excellent agreement for CD4⁺ T-lymphocyte percentages (r=0.9914) and absolute counts (r=0.9697). Linear regression analysis showed a strong association, with r^2=0.9403 for absolute counts. Bland–Altman analysis indicated a mean difference of 61.06 cells/µL (95% CI: -73.91 to 196.04), with most values falling within the confidence limits. Among patients with CD4⁺ T-lymphocyte percentages ≤ 20%, the mean difference was 28.56 cells/µL (95% CI: -61.98 to 119.09).

Conclusion: The single-platform method is comparable to the dual-platform method for analyzing CD4⁺ T-lymphocytes in HIV patients. Both methods demonstrated normal data distribution, confirming statistical robustness. High correlations for percentages and absolute counts ensured consistent and reliable results, while regression analysis indicated strong predictive capability of the single-platform method for dual-platform results. Bland–Altman analysis further confirmed the equivalence of the two methods,

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supporting the reliability of the single-platform approach. Overall, the single-platform method offers a reliable and efficient alternative, reducing variability, workload, and turnaround time in laboratory settings while maintaining analytical accuracy.

Introduction

The Joint United Nations Programme on HIV/AIDS (UNAIDS) has urged nations to intensify efforts to end the HIV/AIDS epidemic, following concerns that HIV services had been neglected during the COVID-19 crisis. If this trend continues, it could result in an estimated 7.7 million deaths from HIV/AIDS over the next decade. In Thailand, during 2017–2018 and amidst the COVID-19 outbreak, an average of 16 new HIV infections per day was reported, largely due to reduced testing and a decline in the number of patients receiving antiretroviral therapy. This rate remains below the UNAIDS target of eliminating AIDS by 2030. Despite this decline, UNAIDS remains concerned about achieving the goal.[1] In 2023, Thailand reported approximately 436,170 people living with HIV, 2,503 AIDS-related deaths, and 4,148 new HIV cases. Key affected populations include men who have sex with men, transgender individuals, people who inject drugs, and individuals engaging in unprotected sexual activities. In Health Region 6, about 50,090 people were living with HIV, with 655 new cases and 1,458 AIDS-related deaths. Chonburi Province reported the highest number of new cases (~360) and the largest number of people living with HIV (~18,226). In comparison, Trat Province reported 18 new HIV cases and 2,302 people living with HIV.[2] These statistics underscore the need for ongoing diagnostic testing and treatment.

$CD4^+$ T-lymphocyte testing measures both the absolute count and the percentage of $CD4^+$ T-lymphocytes in blood. Flow cytometry has been developed as a diagnostic tool for determining $CD4^+$ T-lymphocyte counts.[3] This system functions by detecting cells in suspension as they pass through a laser beam, where light scattering and fluorescence signals emitted by labeled cells are detected by sensors and analyzed by computer systems.[4] Absolute $CD4^+$ T-lymphocyte counts are critical for disease prognosis, clinical decision-making, and monitoring antiretroviral therapy (ART) outcomes.[5] Current measurements typically require data from both flow cytometers and blood cell analyzers. The latter provides complete blood count (CBC) parameters, including white blood cell (WBC) count and lymphocyte percentage (% lymphocyte), which are incorporated into $CD4^+$ percentages calculations.[6] However, reliance on two instruments often leads to delays, particularly during urgent testing or when CBC workloads are high. Accuracy may also be compromised by differences in analyzer models or testing methodologies. Testing costs are approximately 23 THB per CBC (government charge: 130 THB) and 125 THB for $CD4^+$ testing (government charge: 900 THB).[7,8] Physicians requiring both absolute and percentage $CD4^+$ T-lymphocyte results must therefore request additional CBC tests, unnecessarily increasing costs.

International studies have explored alternative methodologies for calculating absolute $CD4^+$ T-lymphocyte counts without reliance on CBC results. The single-platform method, which relies solely on flow cytometry, has been introduced as an alternative to the conventional dual-platform approach. This method offers advantages such as simplified workflows, reduced costs, and minimized errors associated with multi-instrument dependency.[9]

In Thailand, research institutions and major hospitals equipped with flow cytometers have begun evaluating single-platform and dual-platform methods.[10] These assessments focus on accuracy, precision, processing efficiency, and cost-effectiveness. The single-platform method employs polymer beads of known size and concentration, which are mixed with patient samples to determine $CD4^+$ percentages. Through light scattering and fluorescence detection, flow cytometers directly measure $CD4^+$ T-lymphocyte counts without reliance on WBC counts and % lymphocytes, thereby streamlining the analytical process.[11] Successful implementation of single-platform testing requires skilled personnel, high-precision equipment, and meticulous sample preparation to ensure accurate and reliable results.[12] Although both international and domestic studies indicate promising outcomes, single-platform methods are not yet widely accepted due to limited research validating their reliability. Given these potential benefits, the present study aims to compare single-platform and dual-platform methods for measuring both absolute counts and percentages of $CD4^+$ T-lymphocytes.

Materials and methods

Sample collection and preparation

A total of 865 whole blood samples (3 mL, EDTA-anticoagulated) were collected from HIV-infected patients in Trat Province between April 1, 2024, and July 31, 2024, for the analysis of $CD4^+$ T-lymphocyte percentages and absolute counts. All sample collection and analyses were conducted at the Division of Virology, Department of Medical Technology, Trat Hospital. White blood cell counts and lymphocyte percentages were obtained using a semi-automated hematology analyzer UniCel DxH 900 (Beckman Coulter, USA) while $CD4^+$ T-lymphocyte counts and percentages were measured with a flow cytometer Cytomics FC500 (Beckman Coulter, USA). All analytical procedures were validated through rigorous quality control measures.

For immune-phenotyping, each EDTA-treated whole blood sample was stained with the monoclonal antibody CYTO-STAT® triCHROME™ CD45-FITC/CD4-RD1/CD3-PC5 (Beckman Coulter, USA). The immunophenotyping procedure involved mixing 10µL of antibody with 100µL of whole blood in a 12×75 mm plastic tube,

followed by thorough mixing using a vortex mixer. The stained samples were then processed using the TQ-Prep™ Workstation and the IMMUNOPREP Reagent System (Beckman Coulter, USA) for automated sample preparation. Subsequently, the prepared samples were analyzed by flow cytometry using two distinct analytical methods.

Method 1: Dual-platform technique

A total of 100µL of EDTA-treated whole blood was stained with 10µL of CYTO-STAT® triCHROME™ reagent (Beckman Coulter, USA). The samples were incubated for 10 minutes at room temperature (20–25°C) in the dark to allow adequate antibody binding, ensuring reproducibility and standardization. Red blood cells were lysed using the lyse–no-wash protocol with the TQ-Prep™ Workstation. The prepared samples were then analyzed on a flow cytometer Cytomics FC500 (Beckman Coulter, USA). The gating strategy for identifying lymphocyte populations and $CD4^+$ T-lymphocyte subsets is shown in Figure 1.

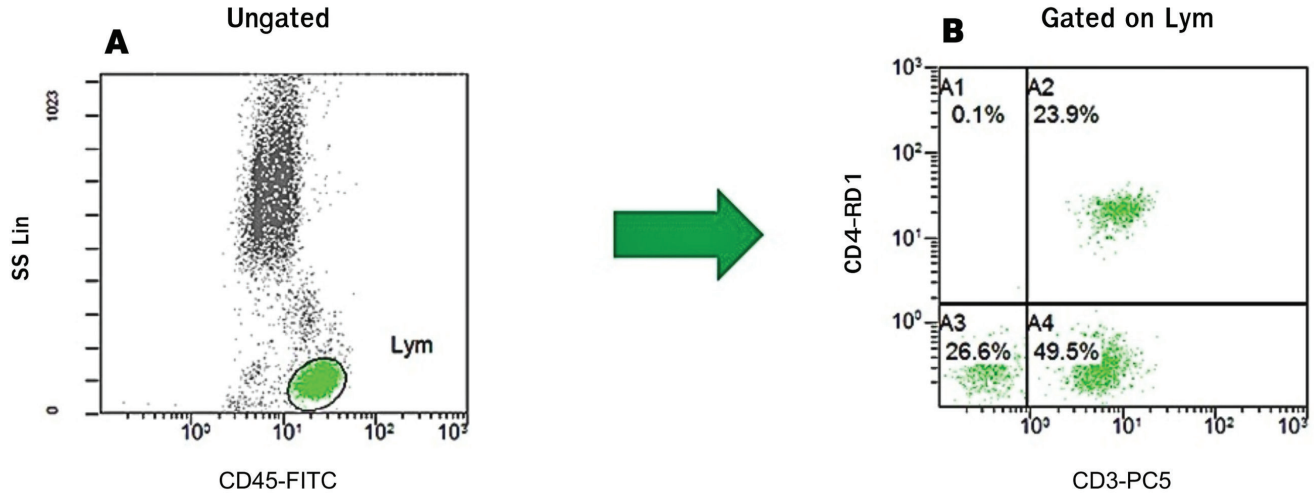

Figure 1. *CD45/SSC and CD3/CD4-based gating strategy for dual-platform $CD4^+$ T-lymphocyte enumeration. A: lymphocyte populations were gated by plot of CD45 against SSC. Lymphocytes are low side scatter and have high CD45, B: $CD4^+$ T-lymphocyte (%) in lymphocyte populations were analyzed by plotting CD3 against CD4. $CD4^+$ T-lymphocyte are positive with CD3 and CD4 (A2 quadrant). Absolute $CD4^+$ T-lymphocyte count derived from % $CD4^+$ T-lymphocyte x %lymphocyte x WBC count/10,000 (%$CD4^+$ T-lymphocyte from flow cytometer, %lymphocyte and WBC count from hematology analyzer).*

Method 2: Single-platform technique

CD4⁺ T-lymphocytes were stained using the same protocol as in the dual-platform technique. After red blood cell lysis, 100 µL of Flow-Set™ Fluorospheres (Beckman Coulter, USA) was added to an equal volume (100µL) of blood sample. The prepared samples were then analyzed on a flow cytometer Cytomics FC500 (Beckman Coulter, USA). The bead-based gating strategy used for absolute CD4⁺ T-lymphocyte enumeration is presented in Figure 2.

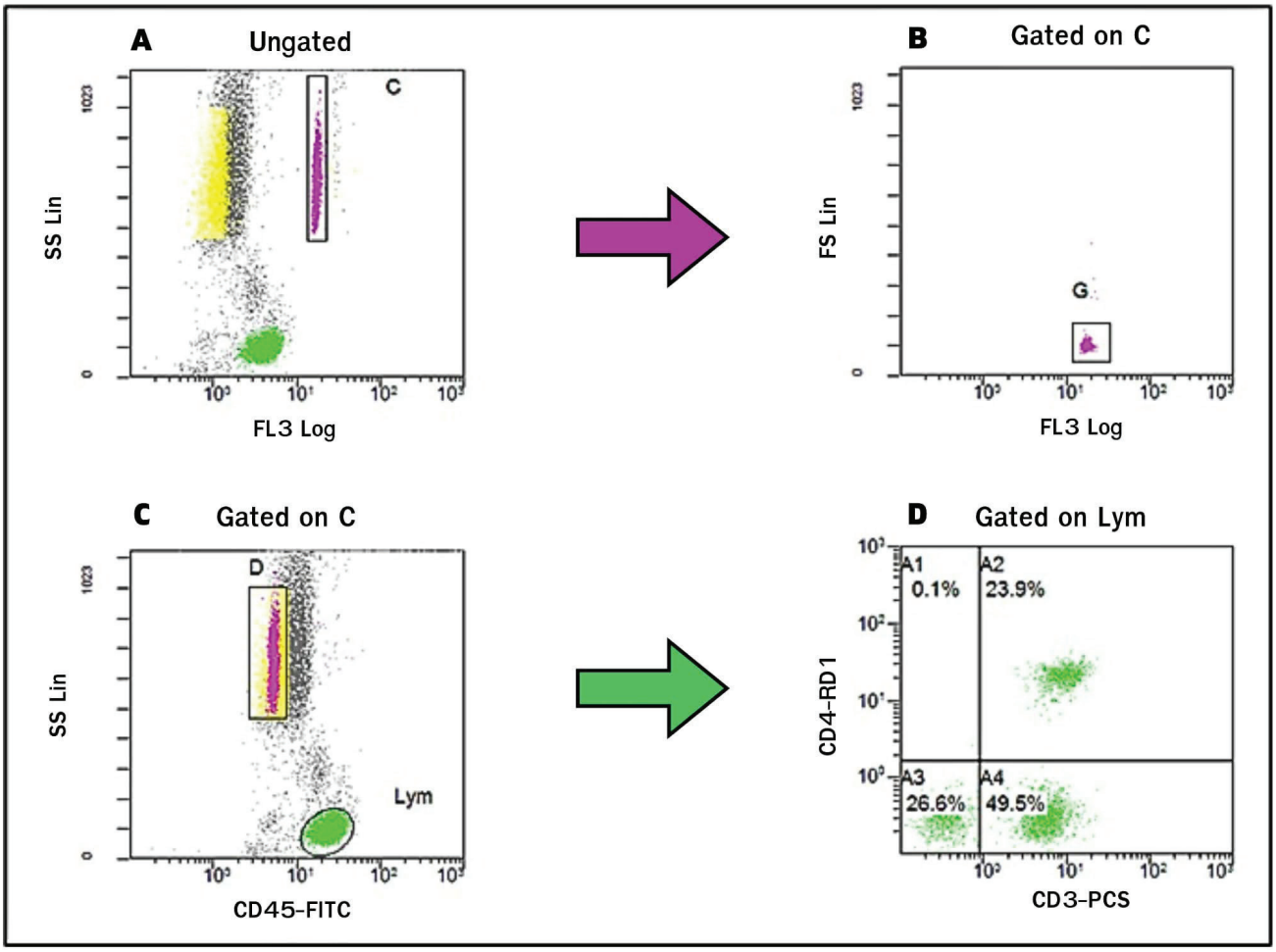

Figure 2. Bead-based gating procedure for absolute CD4⁺ T-lymphocyte enumeration using Flow-Set™ Fluorospheres. *The beads contain fluorescent polystyrene particles that emit at 525-700 nm when excited at 488 nm. The fluorescence signal was detected through the FL3 (ECD) channel using a 610-nm long-pass filter. A: Flow-Set™ Fluorospheres were primary gated by plot of SSC against FL3 (gate C). The fluorospheres from cell events could be separatedroughly, B: only singlet beads were gated from gate C events by plot of FSC against FL3 (gate G), C: lymphocyte populations were gated by plotting CD45 against SSC. In gate D, the pink events are Flow-Set™ Fluorospheres. (D) %CD4⁺ T-lymphocyte cell events in A2 quadrant were interested. Absolute CD4⁺ T-lymphocyte count derived from (CD4⁺ T-lymphocyte events x bead concentration) / bead events.*

Data analysis

Statistical analyses were conducted using Microsoft Excel 2013. For each variable, the mean and SD were calculated. Normality of data distribution was assessed using the Kolmogorov-Smirnov test prior to performing correlation and linear regression analyses. The correlation between absolute CD4⁺ T-lymphocyte counts and percentages obtained from the dual-platform and single-platform methods was evaluated using Pearson's correlation coefficient (r). Linear regression analysis was employed to examine the linear relationship between the absolute CD4⁺ T-lymphocyte counts obtained from both methods. Additionally, Bland-Altman plots were used to assess the agreement between the two methods. The results were presented as the mean bias and limits of agreement (LOA) to determine whether the methods could be used interchangeably.

Results

The percentage and absolute counts of CD4⁺ T-lymphocytes were measured in 865 blood samples from patients using the single-platform volumetric flow cytometry method with the FC-500 flow cytometer. The results were then compared with those obtained using

the standard dual-platform flow cytometry method in conjunction with the semi-automated blood cell analyzer UniCel DxH 900. The findings of this study are presented as follows.

Data distribution testing

The results of the data distribution analysis are presented in Table 1.

As summarized in Table 1, all parameters followed a normal distribution (Kolmogorov-Smirnov test, $p>0.05$). A normality distribution analysis was conducted on the percentage and absolute values of $CD4^+$ T-lymphocytes obtained from HIV patients in Trat Province who submitted samples to the Medical Technology Department at Trat Hospital. The Kolmogorov-Smirnov (K-S) test was applied to assess data normality. The K-S test values for single-platform $\%CD4^+$, dual-platform $\%CD4^+$, single-platform absolute $CD4^+$ T-lymphocyte count, and dual-platform absolute $CD4^+$ T-lymphocyte Count were 0.153, 0.109, 0.308, and 0.207, respectively. All values exceeded the critical threshold at the 0.05 significance level, confirming that the data for all four parameters followed a normal distribution.

Table 1. Statistical analysis and Kolmogorov–Smirnov test of samples.

Measurement parameter	N	Mean	SD	Kolmogorov–Smirnov test (K-S Test)	*p* value (N>40)
Single-platform $\%CD4^+$	865	25.0229	8.9762	0.153	0.05
Dual-platform $\%CD4^+$	865	25.0090	9.0796	0.109	0.05
Single-platform absolute $CD4^+$ count	865	490.00	241.46	0.308	0.05
Dual-platform absolute $CD4^+$ count	865	551.18	269.69	0.207	0.05

The correlation analysis

Correlation analysis was conducted to evaluate the relationship between single- platform $\%CD4^+$, dual-platform $\%CD4^+$, single-platform absolute $CD4^+$ T-lymphocyte count, and dual-platform absolute $CD4^+$ T-lymphocyte Count. The correlation coefficients (r) obtained from the statistical tests are presented in Table 2.

Table 2. Pearson correlation coefficient analysis of single and dual-platform methods.

Variable	Single-platform $\%CD4^+$	Single-platform absolute $CD4^+$
Single-platform $\%CD4^+$	1.0000	
Single-platform absolute $CD4^+$	0.7315	1.0000
Dual-platform $\%CD4^+$	0.9914	0.7233
Dual-platform absolute $CD4^+$	0.7430	0.9697

The statistical analysis demonstrated a strong correlation between the two methods for measuring the percentage of $CD4^+$ T-lymphocytes, with a correlation coefficient (r) of 0.9914. Similarly, the analysis of absolute $CD4^+$ T-lymphocyte counts using both methods exhibited a very high correlation, yielding a correlation coefficient (r) of 0.9697. The correlation coefficients are presented in Table 2 and illustrated in Figure 3**,** showing excellent agreement between single- and dual-platform methods.

The linear regression

Statistical analysis using linear regression demonstrated a strong correlation between the single-platform absolute $CD4^+$ T-lymphocyte count and the dual-platform absolute $CD4^+$ T-lymphocyte Count, with an R^2 value of 0.9403. The regression analysis demonstrated a strong relationship between the two methods, as illustrated in Figure 3B.

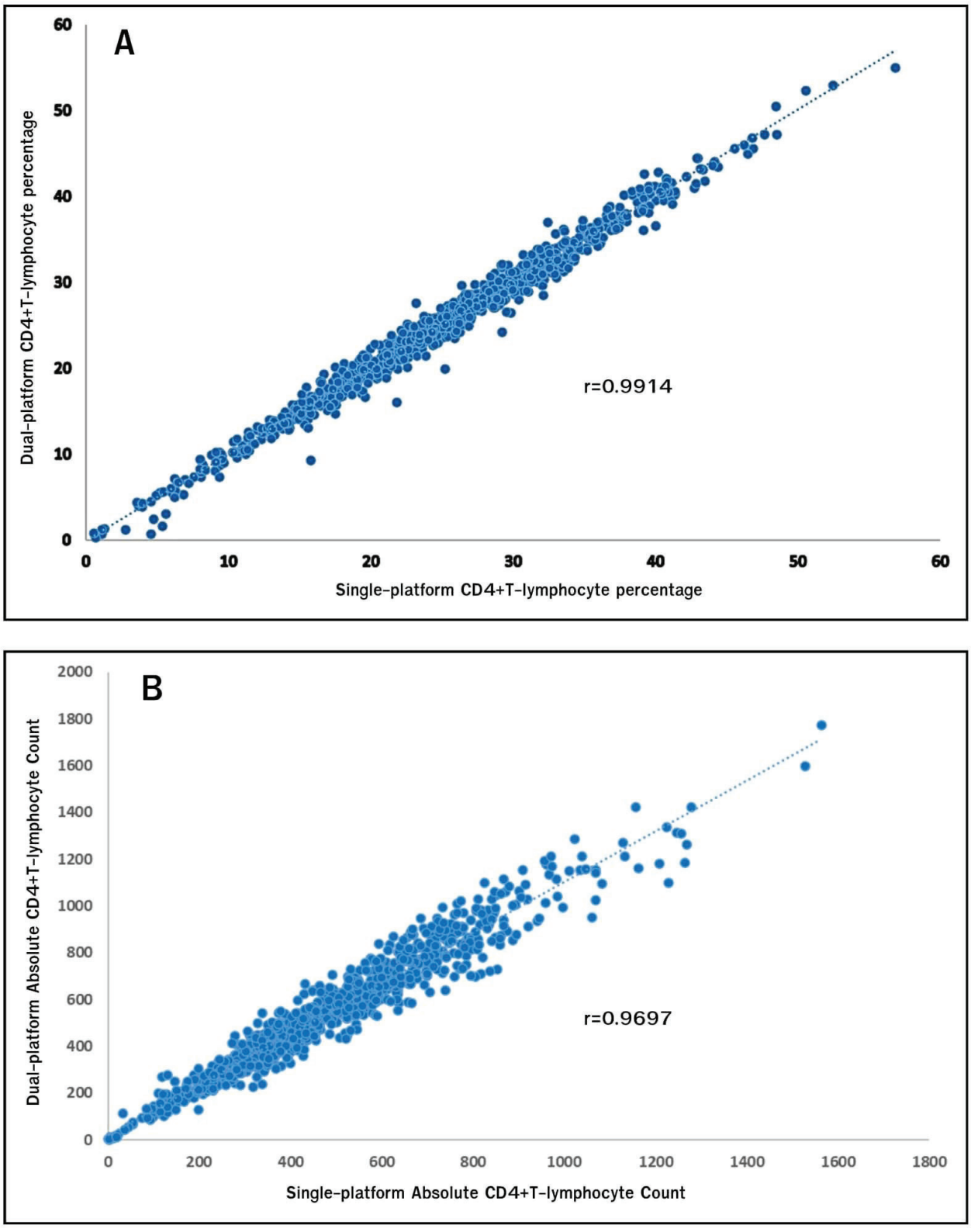

Figure 3. ***Correlation analysis of CD4⁺ T-lymphocyte values obtained using the single-platform and dual-platform techniques.*** *A: scatter plot illustrating the correlation between CD4⁺ T-lymphocyte percentages measured by both methods, B: scatter plot depicting the relationship between absolute CD4⁺ T-lymphocyte counts from the two analytical approaches.*

Bland-Altman plot analysis

Analysis of the single-platform and dual-platform absolute CD4⁺ T-lymphocyte counts using the Bland-Altman method revealed that the mean differences for both the percentage and absolute values of CD4⁺ T-lymphocytes were predominantly within the 95% confidence interval (95% CI). For the absolute CD4⁺ T-lymphocyte count, the mean difference was 61.06 cells/μL, with a 95% CI ranging from -73.91 to 196.04 cells/μL. The Bland-Altman plot in Figure 4 confirms that most values lie within the 95% confidence interval, with a mean bias of 61.06 cells/μL.

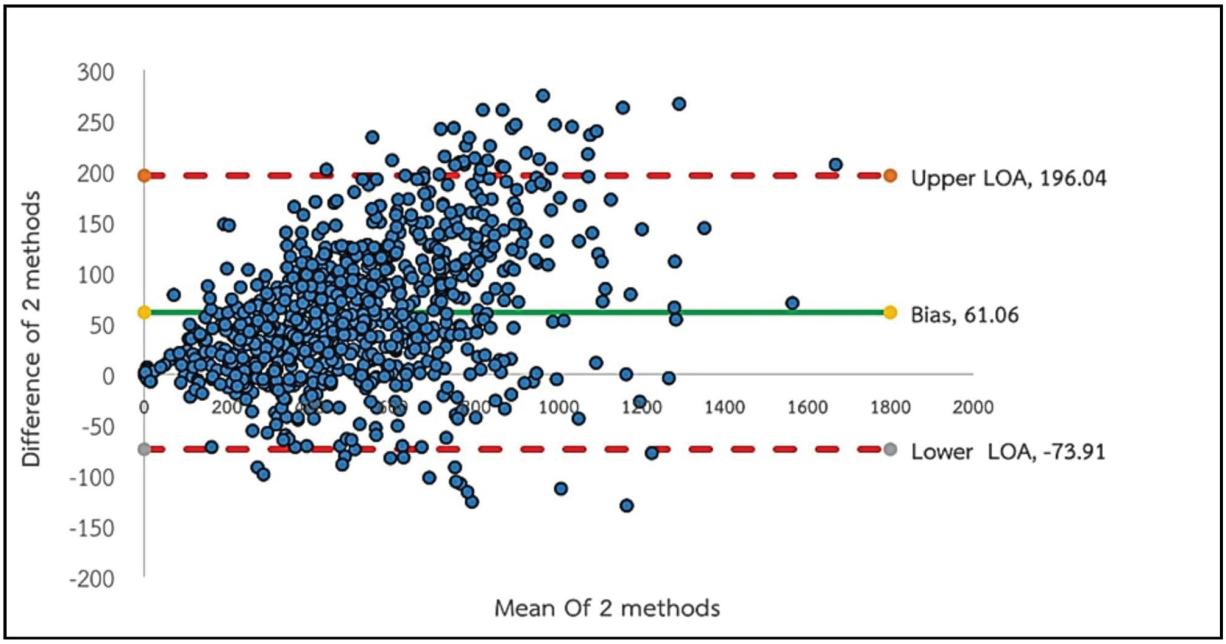

Figure 4. *Bland-Altman plot diagram analysis of absolute CD4⁺ T-lymphocyte count from single and dual-platform methods (N=865).*

When considering the analysis of absolute CD4⁺ T-lymphocyte count from a sample of 246 participants with a CD4⁺ percentage ≤20%, who represent a target group requiring close monitoring and timely treatment, using a Bland-Altman plot, the mean difference was found to be 28.56 cells/μL (95% CI: -61.98 to 119.09) For patients with CD4⁺ T-lymphocyte percentages ≤20%, the Bland-Altman analysis (see Figure 5) revealed a smaller mean difference of 28.56 cells/μL, with most results remaining within acceptable limits when compared to the normal CD4⁺ T-lymphocyte range (500-1500 cells/μL).[13]

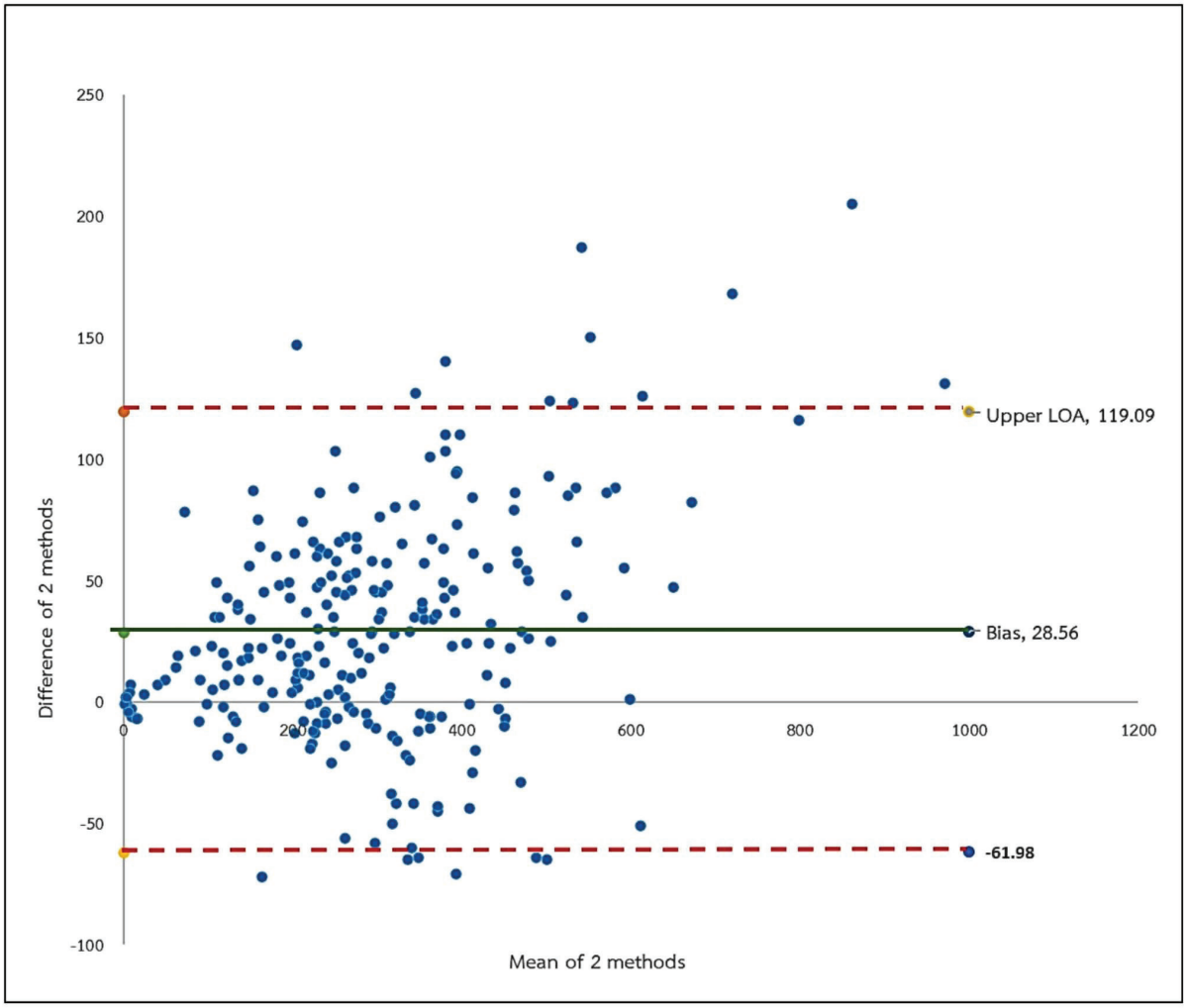

Figure 5. *Bland-Altman plot diagram of absolute CD4⁺ T-lymphocyte count analysis from single and dual-platform methods when %CD4⁺ ≤20% (N=246).*

Discussion

In Thailand, CD4⁺ T-lymphocyte enumeration remains a cornerstone of HIV patient management, guiding decisions on initiating antiretroviral therapy and preventing opportunistic infections. The conventional dual-platform technique, which integrates results from hematology analyzers and flow cytometers, has long been regarded as the standard approach. However, its reliance on multiple instruments, high costs, the requirement for skilled personnel, and stringent quality control procedures limit its accessibility in smaller hospitals and resource-limited laboratories. Moreover, variability arising from differences in reagents, instrument models, calibration methods, or operator performance can further undermine result reliability.

The single-platform method was evaluated in this study as a practical and efficient alternative. By relying solely on flow cytometry, this approach eliminates the need for complete blood count testing, thereby streamlining workflow, reducing analytical variability, shortening turnaround times, and lowering laboratory workload while maintaining high accuracy. International experience with volumetric flow cytometry supports its reliability, and in this study, polymer beads were used as standardized calibration materials. Their defined size and concentration served as a consistent internal reference, enhancing precision and reproducibility in CD4⁺ T-lymphocyte enumeration.

From an economic perspective, the single-platform method offers distinct advantages. The estimated cost per test was approximately 125 THB, compared to 148 THB for the dual-platform approach, representing a cost reduction of approximately 15.5%. This cost-saving is particularly significant in resource-limited settings, where efficient use of diagnostic resources is essential for sustaining HIV care programs. The robustness of this approach is further supported by the strong correlation and agreement observed between the two methods, as illustrated in Table 2 and Figures 3-5, which together provide consistent evidence confirming the reliability of the single-platform technique.

Taken together, these findings suggest that the single-platform method yields results that are clinically comparable to those obtained with the dual-platform approach, while also providing operational and economic

benefits. Nonetheless, challenges remain, including the relatively high cost and limited availability of reagents, as well as the need for adequately trained personnel to ensure consistent implementation. Addressing these limitations will be essential for broader adoption.

Conclusion

In conclusion, the single-platform method represents a reliable, accurate, and cost-effective alternative for $CD4^+$ T-lymphocyte monitoring in HIV-infected patients. Its implementation has the potential to enhance laboratory efficiency, expand diagnostic accessibility, and support national strategies for strengthening HIV/AIDS management in Thailand.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Ethical approval

The study was approved by the Trat Provincial Human Research Ethics Committee (Approval No. **013/2567**, dated March **29**, **2024).**

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