Immune status monitoring of HIV/AIDS patients in resource-limited settings: a review with an emphasis on CD4$^+$ T-lymphocyte determination

Kovit Pattanapanyasat[1,2]

Summary

CD4 T-lymphocytes play a vital role in maintaining the integrity of the human immune system. They are also the primary target cells for human immunodeficiency virus (HIV). The progressive depletion of these cells eventually results in weakening of the host's immune ability to fight against any pathogen, thus rendering the host susceptible to infections and leading ultimately to death of patients in the terminal stage of acquired immune deficiency syndrome (AIDS). Although several clinical and laboratory parameters have been used for monitoring disease progression and the effectiveness of HIV antiretroviral therapy (ART), it is the simple measurement of CD4$^+$ T-lymphocytes that remains the single and most important parameter for management of HIV-infected patients in resource-limited settings. To date, flow cytometer is considered to be the most accepted technology for both percentage and absolute CD4$^+$ T-lymphocyte determination because of its accuracy, precision and reproducibility. However, flow cytometer based CD4 testing is relatively expensive, complex and thus technically demanding. Simple innovative approaches applicable to the conventional flow cytometric system and new technologies have been successfully developed to increase cost saving especially for use in resource-challenged settings. Principles of the existing dual- and single-platform approaches as well as several affordable CD4 measurement technologies are discussed along with both internal and external quality control systems in the management of laboratories performing CD4 testing. ***(Asian Pac J Allergy Immunol 2012;30:11-25)***

Key words: *AIDS, CD4 testing, CD4 T-lymphocytes, flow cytometry, HIV monitoring, resource-limited settings*

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Introduction

Acquired immune deficiency syndrome (AIDS) is now known to be the greatest pandemic of modern times. The global AIDS epidemic has progressed to the point where current estimates suggest that at least 33 million people have been infected with human immunodeficiency virus (HIV), the virus that causes AIDS; of whom over two thirds are living in developing countries with limited resources.[1] At the end of 2009, there were an estimated 4.1 million people living with HIV in the South and South-East Asia Regions with over 95% of cases having been reported from India, Thailand, and Myanmar. Amongst all intervention strategies applied to curtail the HIV pandemic, reduction of viral load by efficient antiretroviral therapy (ART) has been considered to be a powerful tool. The recent introduction of relatively inexpensive and generic antiretroviral drugs has been an important step in the fight against the HIV/AIDS in resource-constrained settings.[2,3] The significant scale up of the ART program over the past few years has contributed to a 19% decline in deaths among people living with HIV since 2004.[1] For a successful ART program, both virological and immunological monitoring are essential.

As elsewhere in the world, but particularly so in underdeveloped and developing countries, health care systems are under increasing pressure to operate cost-effectively. The costs of ART have continued to drop in many resource-constrained settings due to affordable antiretroviral drugs, but that has not necessarily been the case with the cost

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of monitoring. It is realized that adequate management of the health care of persons infected with HIV in these countries is possible only if ART is accompanied by accessible and affordable laboratory monitoring. Although viral load testing is considered to be more informative for guiding decisions regarding when to implement ART, it is expensive and not readily available in these countries. Laboratory monitoring in the form of $CD4^+$ T-lymphocyte enumeration is therefore the most effective method, as it is a prerequisite for initiation of ART and for assessment of immune competence in treated HIV-infected individuals. For almost two decades, our laboratory has been working on the development of several low-cost methods for enumerating $CD4^+$ T-lymphocytes and has carried out evaluation of many inexpensive CD4 testing systems for HIV monitoring in Thailand and worldwide. The results of these studies constitute the basis of this report.

Flow cytometric determination of percent and absolute number of $CD4^+$ T-lymphocytes

Flow cytometric analysis for measuring both the percentage and the absolute number of $CD4^+$ T-lymphocytes using three- and four-color immune-phenotyping is today the gold standard for the estimation of $CD4^+$ T-lymphocyte counts due to its accuracy, precision, reproducibility and it is also the first choice if a large throughput of samples is required.[4-7] However, flow cytometry based CD4 testing is relatively complex and thus technically demanding. Most CD4 testing techniques require dedicated routine maintenance and costly reagents with specific handling requirements, as well as auxiliary laboratory equipment. Additionally, it is essential that the operators of flow cytometer be sufficiently trained in the technical and biological aspects of CD4 testing. Although the sophisticated flow cytometry system has high set up and running cost nowadays, the introduction of many simple, affordable and portable flow cytometers has facilitated better implementation in resource-limited settings.

There are two flow cytometric approaches commonly utilized today to determine absolute CD4+ T-lymphocyte counts.

Dual-platform approach:

The dual-platform approach uses two instruments to generate absolute $CD4^+$ T-lymphocyte values: a flow cytometer for generating %$CD4^+$ T-lymphocytes among lymphocytes and a hematological analyzer to enumerate the absolute lymphocyte counts.[6-8] An absolute $CD4^+$ T-lymphocyte value is then derived by multiplying the %$CD4^+$ T-lymphocytes by the absolute lymphocyte count. It is important to note that the %$CD4^+$ T-lymphocyte value is a more useful parameter for monitoring HIV infection in pediatric patients. This is because the percentage values vary significantly less than the absolute $CD4^+$ T-lymphocyte values,[9,10] which is the basis for the World Health Organization (WHO) recommendation that the %$CD4^+$ T-lymphocytes be used in the decision-making for ART initiation in infants younger than 5 years of age.[11,12] However, the dual-platform approach has a problem with precision of the absolute $CD4^+$ T-lymphocyte count, which is dependant on deriving the absolute lymphocyte count from the hematological analyzer.[9,13-15] Single-platform flow cytometric technology, on the contrary, is designed to enable determinations of both absolute and percentage $CD4^+$ T-lymphocyte values using a single tube in a flow cytometer.[16-18]

Because the %$CD4^+$ T-lymphocytes is obtained from the reference lymphocyte population, the purity of the lymphocytes is therefore essential. A reliable method for assessing lymphocyte purity and recovery on the basis of the differential CD45 antigen density expression has been developed by Loken et al.[19] and later modified for use in more complicated blood samples.[20] This method is also known as CD45 gating. CD45 is a pan-leucocyte marker expressed at different intensities on each leucocyte population, having the highest expression in the lymphocyte population. In the three-color immunophenotyping technique, three conjugated monoclonal antibodies (mAbs) are used to label blood samples. A commercial cocktail of the mAbs called TriTEST™ is available (Becton Dickinson {BD} Biosciences; San Jose, CA, USA). The TriTEST mAbs are anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-phycoerythrin (PE), and anti-CD45-peridinin chlorophyll protein (PerCP). For those who use the Beckman Coulter flow cytometer, the three conjugated mAbs named Opticlone™ (Beckman Coulter {BC}, Miami, FL, USA) are used, these are anti-CD3-FITC, anti-CD4-PE, and anti-CD45-phycoerythrin-cyanin 5.1 (PC5). For the four conjugated mAbs, BD MultiTEST™ of mAbs to CD3-FITC, CD8-PE, CD45-PerCP, and CD4 Allophycocyanin (APC) or BC Cyto-Stat tetraCHROME™ of mAbs to CD45-FITC, CD4 RD1 (phycoerythrin), CD8 phycoerythrin-Texas Red-x (ECD), and CD3-PC5 are used to label blood

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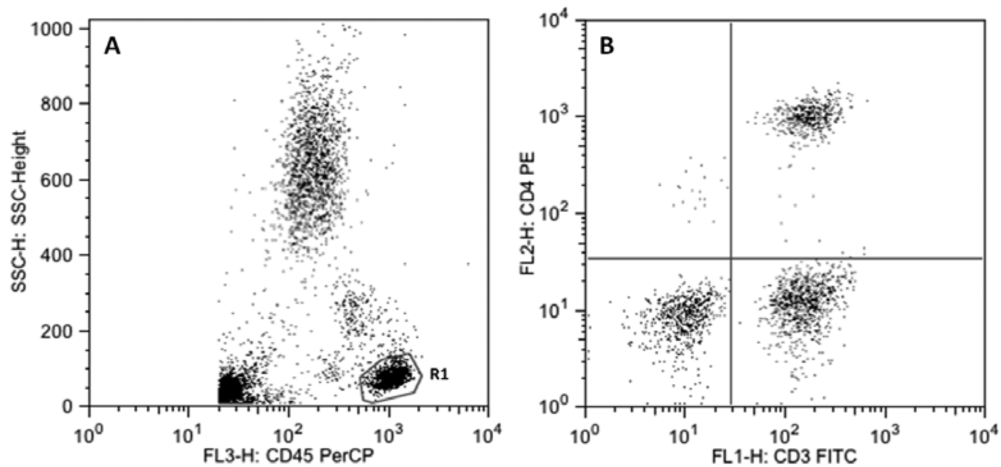

Figure 1. Representative flow cytometric two-parameter dot plots of a stained whole blood sample by BD TriTEST of mAbs to CD3-FITC/CD4-PE/CD45-PerCP three-color reagent. (A) The CD45-PerCP/SSC-H dot plot shows all leucocyte populations with marked CD45 expression in lymphocytes in R1. (B) Determination of % $CD3^+/CD4^+$ T-lymphocytes (upper right quadrant) is obtained from R1 gated cell population.

samples. Following introduction of an immune-stained blood sample with the lyse-no-wash method into the BD flow cytometer, e.g. FACSCalibur^{TM}, the threshold corresponding to the logarithmic (log) CD45-conjugated fluorochrome (PerCP) is set as near as possible to all leucocyte populations in the linear 90° light side scatter (SSC-H) versus log CD45 plot. The lymphocyte gate is then set around lymphocyte population (Figure 1A) and lymphocytes are defined as $CD45^{bright}$ with low SSC-H (R1), cells outside this gate are considered to be monocytes ($CD45^{intermediate}$/$SSC\text{-}H^{medium}$) and granulocytes ($CD45^{low}$/$SSC\text{-}H^{high}$). Once the lymphocyte gate is established, the percentage of double-positive $CD3^+/CD4^+$ T-lymphocytes in the upper right quadrant of a two-parameter dot-pot (Figure 1B) is then automatically generated. If the percentage $CD3^+/CD8^+$ T-lymphocytes is required, another three-color mAb reagent panel of CD3-FITC/CD8-PE/CD45-PerCP for BD flow cytometer or CD45-FITC/CD3-PC5/CD8-ECD for BC flow cytometer is recommended. In the four-color immunophenotyping technique, the lymphocyte gate corresponding to $CD45^{bright}$/$SSC\text{-}H^{low}$ (R1) is set to include all lymphocytes and some non-lymphocyte contaminants (Figure 2A). Next, a second two-parameter dot-plot of SSC-H versus forward light scatter (FSC-H) gated on region R1 is generated with R2 region to include all $CD45^{bright}$ lymphocytes (R2) (Figure 2B). The percentage of double-positives of $CD3^+/CD4^+$ T-lymphocytes (Figure 2C) and % $CD3^+/CD8^+$ T-lymphocytes (Figure 2D) are generated based on the R1 * R2 logical gate events. For the absolute $CD4^+$ T-lymphocyte count, the %$CD3^+/CD4^+$ T-lymphocytes is multiplied by absolute lymphocyte count obtained from the hematological analyzer. It is important to note that the gating setting of the signal discriminator or the signal threshold is critical because the mAbs used by the two commercial systems are conjugated with different fluorochromes and have different fluorescent intensity. Therefore, BD uses a fluorescence signal discriminator whereas BC uses a FSC-H threshold for generating CD45 versus SSC-H. It is worth noting that the CD45 gating technique has several advantages. It is a reliable technique to distinguish lymphocytes in the $CD45^{bright}$/$SSC\text{-}H^{low}$ region even in samples that contain a large amount of non-lymphocytes and debris,[20] which allows the use of the lyse-no-wash staining method. The technique also provides the percentage of lymphocytes among leukocyte populations from the CD45/SSC-H gate, and because no isotype control is required, the cost of an isotype control reagent is avoided. In 2002, the three-color or four-color flow cytometric CD45/ SSC-H gating strategy was adopted with a more practical two-color Pan*Leuco*gating approach.[21,22]

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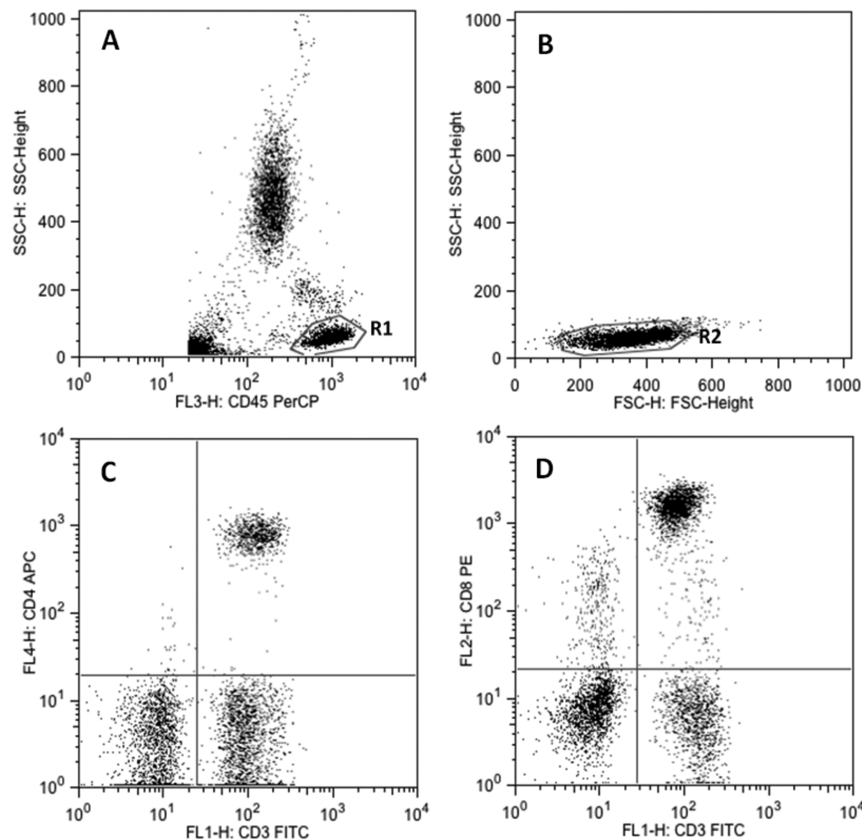

Figure 2. Representative flow cytometric two-parameter dot plots of a stained whole blood sample by BD MultiTEST of mAbs to CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC four-color reagent. (A) The CD45-PerCP/SSC-H dot plot shows all leucocyte populations with marked expression of CD45 in lymphocytes in R1. (B) Pure $CD45^{+}$ lymphocyte population (R2) is obtained from R1 gated cell population using FSC-H/SSC-H dot plot. Both $CD3^{+}/CD4^{+}$ (C) and $CD3^{+}/CD8^{+}$ T-lympho cytes (D) are identified from the logical gating of R1 * R2.

This approach provides both %lymphocytes and $\%CD4^{+}$ T-lymphocytes by advocating the use of the total leucocyte count as the common denominator, in which total leucocyte populations (lymphocytes, monocytes and granulocytes) are identified and gated by their CD45/SSC-H characteristics. Two regions on all $CD45^{+}$/SSC-H leucocyte populations and the $CD45^{bright}/SSC\text{-}H^{low}$ lymphocyte population are set up. The percentage of lymphocytes is obtained from the $CD45^{bright}/SSC\text{-}H^{low}$ lymphocyte population, divided by all leucocyte populations and multiplied by 100. The $CD45^{bright}/SSC\text{-}H^{low}$ lymphocytes in this region are further analyzed for $CD4^{+}$ T-lymphocytes by plotting another $CD4^{+}$/SSC-H plot. $CD4^{+}$ T-lymphocytes are easily distinguished from non-$CD4^{+}$ T-lymphocytes and the percentage of $CD4^{+}$ T-lymphocytes is then obtained as a percentage of total lymphocytes. This CD45-assisted Pan*Leuco*gating approach has been evaluated and accepted in many resource-limited settings since it is simple and more cost-effective as it uses two mAb reagents instead of three or four mAb reagents without sacrificing accuracy and precision.[21-26]

The dual-platform approach requires the results from three separate laboratory tests: the percentage of CD4+ T-lymphocytes from the flow cytometer, the total white blood counts and the percentage of lymphocytes from the hematological analyzer. It is well accepted that the use of a hematological analyzer as the second platform for the white blood count and differential count introduces variable factors into the calculation of absolute $CD4^{+}$ T-lymphocyte values. These are particularly an issue in the inter-laboratory studies that use different hematological analyzers.[27-29] In the individual patient follow up study, the absolute $CD4^{+}$ T-lymphocyte counts obtained from the dual-platform approach contribute to more variability than the $\%CD4^{+}$ T-lymphocyte values (20-33% within-Pearson' Coefficient Variation (CV) versus CV of 10-16%).

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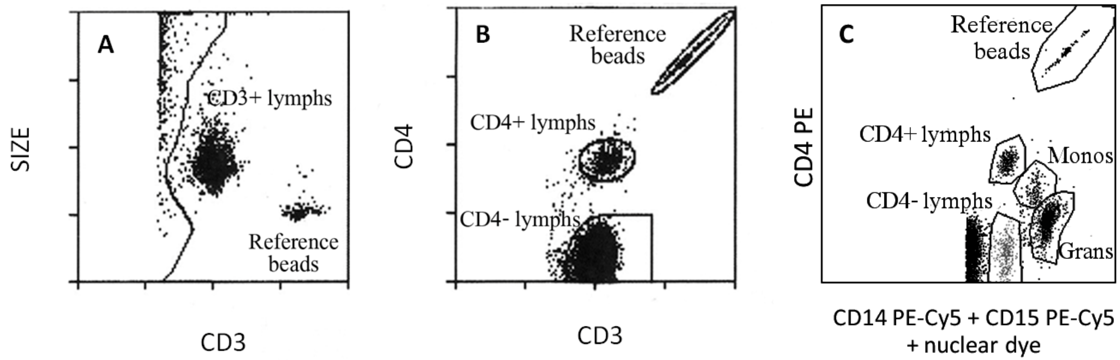

Figure 3. Representative flow cytometric dot plots illustrating BD FACSCount software algorithm of the two-color CD4-PE/CD3-PE.Cy5 (A, B) and the new CD4-PE/CD14-PE.Cy5/CD15-PE.Cy5/Nucleic acid dye system (C).

In addition, the lymphocyte population defined by flow cytometer may not match that of the hematological analyzer. Nevertheless, the dual-platform approach is still recommended in a number of institutional guidelines[6-8] and is widely practiced in many countries including Thailand, as it is cheaper compared to the single-platform approach.

Single-platform approach:

This approach enables absolute CD4$^+$ T-lymphocyte counts to be derived directly without the need for a hematological analyzer. This can be achieved either by counting CD4$^+$ T-lymphocytes in a precisely determined blood volume or by addition of the known number of microfluorospheres or microbeads admixed to a known volume of CD4 immuno-stained blood samples. Importantly, this approach requires a high level of dispensing precision in all pipetting steps and the final dilution factor must be strictly controlled. The use of preprogrammed electronic pipette or reverse pipetting technique is therefore very crucial and most reliable.

At present, the single-platform approach is the preferred technology in an increasing number of laboratories,[30] as it is more reliable and reproducible than the dual-platform approach. The single-platform approach has two options, namely microbead-based technologies and the volumetric technologies. Below are some of these alternatives which have been approved either by U.S. Food and Drug Administration as In Vitro Diagnostics (IVD) devices or by the Conformité Européene (CE) Mark for equipment from the European Union.

FACSCount™

FACSCount is the bench-top microbead-based system from BD.[31] It is the only available microbead-based system that is designed to provide absolute and percentage values of CD4$^+$ T-lymphocytes in no-lyse, no-wash whole blood, which simplifies sample preparation and allows streamlining of the workflow. The instrument is equipped with a green HeNe laser for excitation of two fluorescent parameters: PE and tandem fluorochrome consisting of PE.Cy5. The FACSCount reagent kit is in a ready-to-use twin-tube format. The first tube consist of a mixture of PE and PE.Cy5 conjugated mAbs to CD4 and CD3 T-lymphocytes and a second tube containing PE and PE.Cy5 mAbs to CD8 and CD3 T-lymphocytes. In addition to the mAb reagents, the reagent tubes also contain a known number of fluorochrome labeled microbeads. These reference microbeads function as fluorescence standards for locating the lymphocytes and also as a quantitation standard for determining the cell counts. With this 2-tube reagent kit, the assay delivers absolute CD4$^+$, CD3$^+$, and CD8$^+$ T-lymphocyte counts and a CD4/CD8 ratio, but no %CD4$^+$ T-lymphocytes is provided. Recently, a new BD FACSCount CD4 reagent kit has been provided by BD[31] and has been evaluated as a simple and reliable reagent for determination of both the percentage and absolute CD4$^+$ T-lymphocyte counts.[32] This CD4 reagent kit consisting of a single tube containing a mixture of three mAbs to CD4/CD14/CD15 (conjugated with PE/PE.Cy5/ PE.Cy5, respectively). The mAb to CD14 recognize a human monocyte/macrophage antigen, whereas mAb to CD15 recognizes a human myelomonocytic antigen. Apart from the CD4 reagent, the tube also contains a nuclear DNA fluorescent dye and a known concentration of fluorescent reference microbeads. To assess the accuracy of the FACSCount system, quality control reagents consisting of fluorescent microbeads at three

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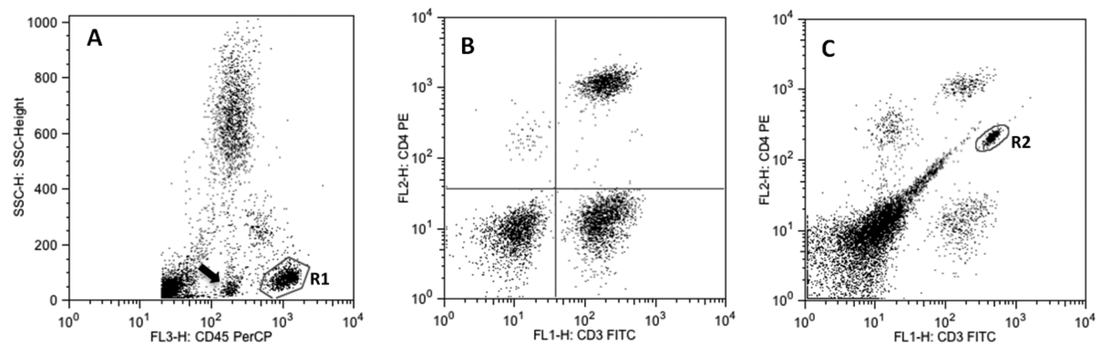

Figure 4. Representative two-parameter dot plots illustrating BD CellQuest software algorithm of the TriTEST mAbs to CD3-FITC/CD4-PE/CD45-PerCP reagent. Lymphocytes in R1 are identified as $CD45^{bright}$/$SSC-H^{low}$ (A). $CD3^+$/$CD4^+$ T-lymphocytes (upper right quadrant) from R1 are obtained by quadrant analysis of CD3-FITC/CD4-PE (B). Glutaraldehyde-fixed chicken RBCs used as reference microbeads are depicted in R2 (C). Arrow in the CD45-PerCP/SSC-H dot plot indicates glutaraldehyde-fixed chicken RBC population.

(for new BD FACSCount CD4 reagent) and four different densities (for BD FACSCount reagent), namely zero (o beads/µL), low (50 beads/µL), medium (250 beads/µL), and high (1,000 beads/µL), are used prior to determining each batch of immuno-stained samples.

In the FACSCount absolute $CD4^+$ T-lymphocyte counting system, following the introduction of an immuno-stained blood sample into the FACSCount system, $CD3^+$ T-lymphocytes ($CD3^+$ lymphs) and reference beads in the threshold gate are automatically generated by the built-in software (Figure 3A). Two elliptical regions and one rectangular gate are automatically set around $CD43^+$/$CD4^+$ T-lymphocytes ($CD4^+$ lymphs), reference beads and $CD3^+$/$CD4^-$ T-lymphocytes ($CD4^-$ lymphs), respectively (Figure3B). After acquiring 30,000 events per sample, the absolute values of $CD3^+$/$CD4^+$ T-lymphocytes and $CD3^+$ T-lymphocytes are calculated automatically by multiplying the ratio of number of $CD3^+$/$CD4^+$ T-lymphocyte events to that of reference beads by the known density of reference microbeads used in the staining tube. If the blood sample is immuno-stained with the BD FACSCount using a new CD4 reagent kit, the built-in BD software algorithm will automatically identify the total number of lymphocyte, the $CD4^+$ T-lymphocyte population, and the reference beads (Figure 3C). Lymphocytes are identified by their DNA fluorescence and size, while monocytes and granulocytes are excluded from the lymphocyte population by their $CD14^+$ and $CD15^+$ antigen expression. Subsequently, the percentage of $CD4^+$ T-lymphocytes is obtained automatically as a percentage of the total lymphocytes ($CD4^+$ and $CD4^-$ lymphocytes). The absolute $CD4^+$ T-lymphocyte count is calculated automatically by multiplying the ratio of fluorescent $CD4^+$ T-lymphocyte events to that of the bright fluorescent bead events by the known number of reference microbeads used in the staining tube.

It should be noted that the dual-platform approach when combined with known reference microbeabs could be used as a single-platform flow cytometric approach for enumeration of absolute $CD4^+$ T-lymphocytes. There is presently three- and four-color automated immunophenotyping software available from many manufacturers, e.g BD MultiSET™ which is used for BD TriTEST and MultiTEST reagents.

Guava® Personal Cell Analyzer (PCA)

The Guava PCA is another bench-top single-platform system known as a microcapillary cytometer developed by Guava Technologies Inc. Hayward, CA, USA. The system is equipped with a 532-nm green diode laser with a forward scatter (FSC) detector and two fluorescence detectors for orange (580 nm) of PE and red fluorescence (675) of PE.Cy5. It is used in combination with two-color Guava EasyCD4™. The reagents consist of two mAbs directly conjugated to PE and PE.Cy5 for CD4 and CD3 antigens. Absolute $CD4^+$ T-lymphocyte counts are obtained by counting $CD4^+$ T-lymphocyte numbers in a precisely determined volume using the lyse-no-wash technique with only 10 µL of peripheral blood plus 10 µL of premixed mAb reagent and 180 µL of lysing/fixing solution. A volumetric control device in the Guava PCA

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system allows a precise count of cell numbers and fluid volume and is regulated by a variable-speed fluid (a stepper motor syringe) pump that does not require sheath fluid. This innovative sheathless-fluidics design based on a microcapillary flow cell means less volume of blood and less volume of reagents (mAbs and lysing solution) are required to perform the assay, hence greatly reducing biohazardous waste disposal requirements and associated costs. For the Guava PCA system, a quadrant is selected and set on the FSC and the PE.Cy5-conjugated $CD3^+$ T-lymphocytes dot plot. After acquisition of at least 2,000 $CD3^+$ T-lymphocytes, a recommended number of events that would satisfy the requisite degree of statistical significance, the double positive $CD4^+/CD3^+$ T-lymphocytes are then automatically identified and obtained in another two-parameter quadrant plot of CD3-PE.Cy5/CD4-PE. An absolute $CD4^+$ T-lymphocyte count is directly obtained from calculating the number of $CD4^+/CD3^+$T-lymphocytes divided by the total acquired volume and then multiplied by the dilution factor of 1:20 by using the Guava Cytosoft or EasyCD4 Software Module. For percent $CD4^+$ T-lymphocytes, a Guava EasyCD4%™ reagent kit in combination with the Guava EasyCD4% software module or, more recently, the Guava Auto CD4/ CD4% system which provides absolute $CD4^+$ T-lymphocyte counts, %$CD4^+$ of total lymphocytes and total lymphocyte counts is recommended.[33]

The performance of Guava PCA and its EasyCD4 reagent kit have been evaluated in several laboratories against the standard flow cytometric methods for determination of both absolute and % $CD4^+$ T-lymphocyte values.[34-36] These studies show that the Guava PCA system is simple and easy to use, it has a high throughput of 50 to 100 samples per day, which is compatible with the standard FACSCount system, but it is relatively cheap and its compact size means it can easily be moved between laboratories. However, this system requires the same sophisticated infrastructure facilities and technical support as standard flow cytometer does. In addition, a multicenter comparative study is still needed before this system is implemented in resource-limited settings.

*Partec CyFlow^green^*

The Partec CyFlow^{green} is another single-platform bench-top volumetric flow cytometer made by Partec GmbH, Münster, Germany). The CyFlow^{green} or CyFlow SL, is one of the most simplified models in the CyFlow system family and can be powered from a car battery or by solar panels. The one-parameter CyFlow^{green} SL contains a 532-nm solid-state laser for green excitation. It analyzes the absolute number of $CD4^+$ T-lymphocytes using true volumetric absolute counting of the volume from two tips of two electrodes dipped into the fluid at different levels. The counting is triggered when the higher electrode during the aspiration is no longer surrounded by fluid and when the fluid level falls below the second lower electrode, the counting stops. 20 µL of whole blood is pipetted into a Partec test tube, followed by 10 µL of PE-conjugated mAb to CD4 antigen supplied by Partec. After 15 minutes of incubation, 800 µL of no-lyse buffer, also supplied from Partec, is added to the tube and gently vortex-mixed. When an immune-stained sample is introduced into the system, $CD4^+$ T-lymphocytes and monocytes with granulocytes are distinguished by their bright and dim fluorescent characteristics, respectively. In a histogram plot, the absolute $CD4^+$ T-lymphocytes with high fluorescence appear in a prominent peak at the right of the histogram, whereas the weaker but expression of CD4 antigen monocytes appears to the left of the histogram without any overlap with $CD4^+$ T-lymphocytes. The bright absolute $CD4^+$ T-lymphocyte counts are gated and calculated by using the histogram marker set run by CyFlow FlowMax software. There is also a CyFlow^{green} SL_3 with three-parameter (SSC and two-color fluorescence) flow cytometer when used in combination with the Partec CD4% reagent kit provides both absolute and %$CD4^+$ T-lymphocyte values. The %CD4 reagent kit contains PE and PE.Cy5-conjugated mAb to CD4 and CD45 antigens.

Performance evaluation by several laboratories showed that the CyFlow system performed well in comparison with the performance of the standard single-platform FACSCount or bead-based flow cytometer system.[37-40] The CyFlow system, particularly the CyFlow^{green} or CyFlow SL is a relatively affordable, easy to use technology that is useful for immune status monitoring of HIV-infected patients in resource-limited settings. However, like any other standard flow cytometers, operation of this system requires substantial technical expertise and maintenance. A multicenter validation is also essential to determine whether the CyFlow system can be interchanged with other standard flow cytometers before wide spread implementation in resource-limited settings.

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Other alternative single-platform approaches

Modification of a traditional flow cytometer using glutaraldehyde-fixed chicken red blood cells

Although the single-platform bead-based flow cytometric approach has been the preferred technology for enumeration of absolute CD4$^+$ T-lymphocyte counts as it introduces fewer factors and has a lower intralaboratory variation when compared with the dual-platform approach. The reference microbeads are however more expensive than the cost when using a hematological analyzer in dual-platform approach. An additional cost for the reference microbeads either in the form of a lyophilized pellet (e.g. BD TruCOUNT® tube) or liquid suspension (e.g. BC Flow-Count beads) prevents this single-platform bead-based approach from being widely used in many resource-limited countries. The use of the single-platform volumetric flow cytometric approach, although simple and inexpensive, is impractical in these countries due to the additional cost involved in the procurement of new volumetric flow cytometers to replace existing flow cytometers. Consequently, flow cytometers that use the dual-platform approach are still the technology of choice in many resource-limited countries like Thailand and India despite its many drawbacks including inconvenience, problems with accuracy, and, above all, the wide interlaboratory variability due to the use of different hematological analyzers.[9,27,29] In view of these problems, our laboratory has successfully developed a method using glutaraldehyde-fixed chicken red blood cells (CRBCs) as the counting biobeads in determining the absolute CD4$^+$ T-lymphocyte counts from HIV-infected blood samples.[41] It has been known for a long time that glutaraldehyde-fixed CRBCs or rainbow trout RBCs can be used for flow cytometer calibration. These fixed avian and fish RBCs do not need any fluorochrome staining because they become fluorescent due to the compound formed by aldehyde binding to the amino group in the proteins of the cells. These autofluorescent fixed CRBCs are easy to prepare and 50 times cheaper than the reference microbeads.

When a blood sample stained with the BD TriTEST mAb reagent in combination with our fixed CRBCs is analyzed by flow cytometer, the CD45-PerCP/SSC-H characteristics of lymphocytes, monocytes and granulocytes show no difference when compared with those stained with the TriTEST/TruCOUNT except the fixed CRBCs, which express a discrete population of low to high SSC-H and low PerCP fluorescent intensity (Arrow, Figure 4A). Despite such low PerCP fluorescent expression, these fixed CRBCs show good separation from the lymphocyte population and thus enable a gate region to be set on the lymphocyte cluster. Once this lymphocyte gate is established, the percentage of CD3$^+$/CD4$^+$ T-lymphocytes are then generated by the quadrant analysis of CD3-FITC versus CD4-PE plot (Figure 4B). To determine the absolute number of CD3$^+$/CD4$^+$ T-lymphocytes, another gate region is set around the bright fixed CRBC cluster in another un-gated CD3-FITC/CD4-PE plot (Figure 4C). The ratio of CD3$^+$/CD4$^+$ T-lymphocytes to the fixed CRBC events in this CRBC gate multiplied by the known density of fixed CRBCs is used to calculate the absolute CD3$^+$/CD4$^+$ T-lymphocyte counts per µL of blood using the same formula provided by BD MultiSET software. An evaluation study of our glutaraldehyde fixed CRBCs showed that these fixed CRBCs can easily be integrated for use as substitute for microbeads in the routine flow cytometric single-platform approach.[42] Linear regression analysis on 87 HIV-infected blood samples revealed excellent correlation and high percentage similarity with low mean percent bias of our CRBCs, compared with the standard TruCOUNT beads over the entire range including the clinical relevant range of 0 – 200 CD4$^+$ T-lymphocytes per µL. It is foreseeable that this glutaraldehyde fixed CRBCs will markedly reduce the cost of CD4 testing without sacrificing accuracy and precision, thus potentially allowing more HIV/ADS patients access to CD4 monitoring in resource-limited countries.

Modification of a traditional flow cytometer using flow-rate based method

As mentioned above, the single-platform flow cytometric approach can eliminate the variability that is found in the traditional dual-platform approach.[9,27,29] Such variation caused by different hematological analyzers presents significant difficulties for the implementation of external quality assessment program for flow cytometric immunophenotyping. In 2003 and 2004, a group of researchers at the UK National External Quality Assessment Scheme (UKNEQAS) based in Sheffield, UK developed a new flow-rate based flow cytometric method to use as the single-platform approach.[43,44] The new method offers several advantages with respect to sample manipulation. It does not require additional reagents and the staining step and

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no additional cost for the reference microbeads. Moreover, it can be applied to any commercially available flow cytometer. In an effort to address the issue of finding a more economical, accurate and reproducible method for enumerating CD4+ T-lymphocyte counts in the developing countries, e.g. Thailand, our laboratory has therefore taken the lead in validating this alternative non bead-based single-platform flow-rate approach to see whether it could be implemented when using old flow cytometer instruments.[45,46] The results showed that this flow-rate approach is a promising and robust method for performing absolute CD4+ T-lymphocyte counts with excellent reproducibility and comparability to the bead-based approach. Data do not vary in relation to the years of service among a group of old flow cytometers which were tested. Nevertheless, there are some limitations to this method and challenges for implementation. Operation and quality control of the flow cytometer with respect to the constant flow rate are essential as the method requires substantial technical expertise and good maintenance of the instrument.[45]

Non-flow cytometric technologies for determination of CD4+ T-lymphocytes

Microchip with microscopic-based technology

In recent years, the cost of ART per patient per year has significantly reduced due to the introduction of relatively inexpensive and generic antiretroviral drugs. Overall, the number of people receiving therapy has grown 13-fold with more than 5 million people from underdeveloped and developing countries are benefitting from this scaling up of access to ART programs.[1-3] However, the cost for immune status monitoring of HIV-infected patients in the form of CD4+ T-lymphocytes in these low- and middle-income countries remains too expensive. Although some new dedicated flow cytometers are less expensive, the costs for the instruments, infrastructure and the assays are yet not affordable for many of these countries. Furthermore, the operation and maintenance of a flow cytometer requires well-trained personnel as well as other auxiliary laboratory equipment. Manual magnetic bead-based microscopic methods, e.g. BC Cytosphere CD4 count[47] and Dynabeads T4-T8,[48] though inexpensive, are extremely labor intensive, less accurate and have a lower throughput than the traditional flow cytometric approach. These two manual methods will not be discussed in this report.

It is well known that many HIV/AIDS patients in the low-income countries have to travel from rural areas to the testing centers. Thus, the decision to initiate chemotherapy or clinical staging are often made solely on the clinical presentation, which requires clinical acumen and time, both of which are lacking in resource-poor settings because of the limited numbers of clinicians. If, however, there are laboratories in rural areas where testing with a flow cytometer is available, they still require effective networking and logistics because the patients or the blood samples have to be referred, often over long distances and it can take days or weeks before the test process is complete and the results are reported. An ideal CD4 testing system in rural clinics would be a point-of-care (POC) system that is simple, low-cost, robust, and independent of any laborious equipment. Above all, it should reliably provide results with short turnaround times. To meet these requirements, a new microchip-based CD4 counting technology has been developed by the University of Texas, USA for monitoring of HIV/AIDS patients.[49] In this system, blood cells are immuno-stained with two mAbs directly conjugated to AlexaFluor 488- and AlexaFlour 647-conjugated mAbs to CD4 and CD3. Images of the labeled cells captured on membrane are obtained and analyzed by using microscopic optics with the type of charge-coupled device (CCD) developed for digital camera technology. An associated computer algorithm software will then convert the raw digital image into absolute CD4+ T-lymphocyte counts and % CD4+ T-lymphocytes. Such a microchip approach has been validated both in the USA and Botswana and showed good agreement with those from the standard BD flow cytometer bead-based system. Later, these groups of researchers along with others have modified this microchip technology using a microfluidic chamber with internal known volume containing biotinylated-avidin anti-CD4 mAb immobilized on the chamber wall,[50] or PE-conjugated mAb to CD4 antigen and the images are analyzed with a microscopic scanning cell counter.[51,52]

Image cytometer-based technology

The single-platform image cytometer for absolute enumeration of CD4+ T-lymphocytes in whole blood has been developed by a group of researchers led by Professors Jan Greve and Leon W.M.M. Terstappen at the University of Twente, The Netherlands.[53] There are several models of such image cytometers, depending on the staining reagents used. All staining schemes are based on

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CD3/CD4 antibody-specific immunomagnetic separation of positive target cells from whole blood samples. In one of the three-color mAb staining schemes, the white blood cells are immuno-stained with FerroFluid conjugated mAb to CD3 antigen, PE-conjugated mAb to CD4 antigen, and PerCP-conjugated mAb to CD8 antigen. FerroFluid-CD3$^+$ T-lymphocytes with PE or PerCP of CD4$^+$ or CD8$^+$ T-lymphocytes will be immunomagnetically attracted to the analysis chamber. Images of CD4$^+$ and CD8$^+$ T-lymphocytes will be captured by CCD camera with different optical filters and analyzed using an image analysis algorithm. The absolute number of CD3$^+$/CD4$^+$ T-lymphocytes will be obtained from the three images divided by the blood volume corresponding to the three captured images at three different positions. This image cytometer has been evaluated by several laboratories including ours and compared with the standard single-platform bead-based flow cytometric approach, i.e. BD FACSount. Because of its good precision, accuracy and linearity for CD4$^+$ T-lymphocyte enumeration during performance evaluation in The Netherlands,[54,55] and a good correlation with low percent bias when evaluation was performed at the clinical site in Thailand,[56] this single-platform image cytometer is a good candidate for POC CD4$^+$ T-lymphocyte enumeration in resource-limited settings. However, a multi-center comparative study is still recommended if this system is implemented in settings with deficiencies in laboratory infrastructure.

Another POC CD4 counting system called PIMA™ CD4 test has been developed and commercially launched by Alere, Waltham, MA, USA.[57] The PIMA CD4 analyzer is a portable fixed-volume cytometer (size 22 cm x 13 cm x 16 cm) with a walk-away CD4 testing system. It is equipped with miniaturized low-cost multifluorescent imaging optics and a CCD camera, and it is operated using with 100 to 240 V power source or a built-in rechargeable battery. For enumeration of absolute CD4$^+$ T-lymphocytes, twenty-five µL of blood sample taken from venous blood or finger-prick blood is dispensed into a disposable anticoagulant-coated cartridge preloaded with two fluorochrome-conjugated mAbs to CD3 and CD4 antigen. The cartridge is capped and inserted immediately into the analyzer and the test performed. During the 20-minute analysis process, the blood sample is mixed and interacted with the two mAbs in the cartridge. A series of images of the fluorescent labeled CD3$^+$/CD4$^+$ T-lymphocytes in the fixed volume detection chamber are collected and the data are analyzed for the absolute number of CD4$^+$ T-lymphocytes. Results from several recent studies, including ours, showed that the system was relatively easy to operate, as compared with any traditional flow cytometers, and the data were promising with accurate results and low levels of bias comparable with those from the standard flow cytometer methods.[58-60] It is therefore easily integrated into rural clinics where infrastructure is limited or at the standard voluntary and counseling centers, where most of the laboratory testing is usually operated by a paramedic or a nurse. It should be pointed out here that this POC system has not been developed to provide values for the % CD4$^+$ T-lymphocytes count, which is a more useful parameter for monitoring HIV infection in pediatric patients, nor has it been evaluated for running on quality control samples. Like any other newly developed CD4 testing systems, a more complete system assessment and independent multicenter performance studies of this system are needed to ensure that the highest of standards of quality and reliability are met. In an effort to scale up access to rapid and accurate HIV/AIDS monitoring tools, WHO has prequalified the Alere PIMA CD4 Test in November 2011 as one of the WHO's list prequalification diagnostic tests based on an in-depth product assessment, an inspection of the manufacturing facility, and independent multicenter evaluation studies.[61] The WHO's prequalification system will help accelerate the adoption of useful newly developed CD4 systems as they become available.

Performance evaluation of a newly adopted CD4 testing system

When a laboratory adopts a new CD4 testing system, samples should be tested in parallel with a standard method to characterize any systematic differences between the new and the well-established method. Several statistical tools should be used to assess the results of comparison studies. Linear least squares regression analysis is helpful in establishing good correlation between the methods but it is insufficient, particularly when the errors are small. A Bland-Altman bias scatter plot provides a more useful parameter for determining bias between the two compared methods.[62] This is achieved by graphically plotting the difference between each data pair of measurements (method A – method B) on the vertical axis against the average of the pair (method A + method B/2) on the horizontal axis.

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The mean absolute difference or bias and the limit of agreement (equivalent to the mean difference ± 1.96 standard deviation) are then calculated. It is also useful to apply % similarity that allows for a direct comparison of the data from the two paired methods.[63] This can be performed by taking the average between the two compared methods divided by the standard method and multiplying by 100. In clinical studies, such as evaluating CD4 T-lymphocytes for monitoring HIV infection, evaluation studies of newly adopted system or method often conclude that a new method is an acceptable alternative to a standard or reference method based on correlation alone, or based on a 'mean difference' between the two which gives no indication of maximum differences which could be large despite a small mean difference, and is normally different at different tiers of $CD4^+$ T-lymphocyte values even at the clinically relevant range. Therefore performance of misclassification probabilities of upward and downward misclassification is more clinically useful for the method under evaluation.[64] For example, in clinical decision making for initiating ART, treatment is recommended if the absolute $CD4^+$ T-lymphocytes/µL is ≤ 350, but a number of tested samples in the clinical important range of 100 – 250 $CD4^+$ T-lymphocytes/µL may be misclassified as having $CD4^+$ T-lymphocyte value > 350 $CD4^+$ T-lymphocytes/µL and some samples in the 400 – 500 $CD4^+$ T-lymphocytes/µL range may be misclassified as <350 $CD4^+$ T-lymphocytes/µL. Misclassification probabilities accepted by the laboratory may depend on the HIV-infected patients' characteristics, i.e. those who are asymptomatic, as opposed to therapy naïve patients who need a therapeutic decision, etc.

In countries where there are many flow cytometric systems available for CD4 testing, i.e. bead-based, microcapillary and volumetric systems, variations among the different systems are not uncommon due to technical differences in the systems as well as the dispensing of reagents and at all the pipetting steps particularly for all single-platform systems. Our laboratory has compared two volumetric flow cytometers, Guava PCA and Partec $CyFlow^{green}$ with BD FACSCount, BD dual-platform and BD Tritest/TruCOUNT single-platform systems, and showed that the absolute $CD4^+$ T-lymphocyte counts determined by $CyFlow^{green}$ < FACSCount < Dual-platform < BD single-platform < Guava PCA.[65] These variations should be considered if either one of these systems is interchanged with another system.

Quality management in $CD4^+$ T-lymphocyte determination

The use or misuse of $CD4^+$ T-lymphocyte determination has a crucial impact on the effective management of HIV/AIDS patients**.** Quality assurance is therefore an essential element to ensure that the overall quality of the results from testing is reliable, reproducible, traceable, and auditable. Several sets of guidelines addressing quality assurance of CD4 testing have been developed.[6,15-17] It is important that accurate Internal Quality Assessment Scheme (IQAS) and proficiency test or External Quality Assessment Scheme (EQAS) if applicable, be employed by the laboratory. IQAS is used to monitor both sample processing and instrument performance within the laboratory, whereas, EQAS provides an assessment of the entire testing process, including pre- and post-analysis procedures. However, quality procedures for CD4 testing are not commonly provided, possibly because they are less understood than those for other laboratory tests. For IQAS, most CD4 testing laboratories in resource-limited settings do not have access to IQC materials as they are purchased separately from CD4 testing reagents and often considered to be unnecessary and expensive. It is hoped that inclusion of IQC materials in procurement lists for CD4 reagents may help improve laboratory performance. For EQAS, participation in most of the international EQAS requires substantial funding to cover the cost of participation fees and carriages. There are several international EQA programs, i.e. UKNEQAS,[66] in which laboratories have to pay for participation. The high cost of such programs has been a major burden, making its implementation in resource-limited countries difficult. However, there are some free EQA programs such as the Canadian-based Quality Assessment and Standardization for Immunological measures relevant to HIV/AIDS (QASI)[67]. In spite of their irregular schedules; they are useful and cost saving. There are also some regional EQA programs, i.e. African Regional External Quality Assessment Scheme (AFREQAS)[68] which is jointly organized by the QASI and the WHO. Another regional EQA program in Asia is our laboratory COE EQA program.[69] This program has been established since 2003, and has two main objectives. Firstly, it must conduct a low-cost EQA and

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standardization of flow cytometer CD4 T-lymphocyte determinations in HIV/AIDS patients in Thailand and other Southeast Asian countries. Secondly, as an EQA provider, it should provide assistance to countries involved in flow cytometer laboratories' performance evaluations and incorporate educationally and scientifically-based schemes in order to monitor their improvement. Our COE program is a free program originally supported by Thailand National AIDS Program and later by the Thailand National Health Security Office. Towards the end of 2011, there were 50 EQA trials with more than 220 participating laboratories throughout Thailand and neighboring countries. This program has received national recognition and is becoming increasing important as Thailand and other Southeast Asian countries scale up their national programs that provide access to ART for people living with HIV/AIDS.

Conclusion

Flow cytometry is considered to be an important tool in assessing response and failure of HIV-infected patients to ART. However, this is not the case in many resource-constrained countries due to its complexity and high cost relative to the very basic needs of laboratories servicing. Several affordable flow cytometers and non-flow cytometric POC systems based on image capture technologies have been developed and probably will become available in the very near future. These achievements should help alleviate the burden that has already adversely affected the social, economic and health fabric of the vast majority of countries affected by HIV/AIDS epidemics. The choice of the new technologies must comply with the international standards, as different technologies are associated with different performance characteristics. POC CD4 devices will have to be simple and be able to run the QC samples. It is also important for CD4 testing laboratories to participate in external quality control program to confirm the reliability of their results and to ensure the success of the ongoing global scale up of the ART program.

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