Since the first case of a HIV-infected high-risk male subject was recognized in 1981, HIV infection has reached pandemic proportions in many countries worldwide. According to the World Health Organization (WHO) report, more than five million people acquired HIV in 2003, bringing the total number of persons living the HIV to approximately 42 millions. About 70 per cent of these infected people live in Sub-Saharan Africa and Asia. It is estimated that if not treated, 3 million people will die every year of HIV/AIDS. Projections for the next 10 yr suggest that the situation will become even more serious, with a possible 100 million infected people worldwide. This situation not only represents a serious human catastrophe, but also dramatically impacts on the economic and health fabric in countries with high HIV prevalence. Amongst all intervention strategies applied to curtail the HIV pandemic, reduction of viral load by efficient antiretroviral therapy has been considered to be a powerful tool. In India, an introduction of inexpensive and generic antiretroviral therapy (ART) has been initiated by National AIDS Control Organization (NACO). For the successful inexpensive treatment an affordable
laboratory monitoring is utmost essential. The enumeration of CD4+ T-lymphocytes in the peripheral blood is an essential tool for the laboratory monitoring of HIV infected patients for the progression of disease and for the assessment of outcome of antiretroviral treatment.

What are CD4+ T lymphocytes?

The CD4+ T lymphocytes; also known as T helper cells (CD4+ T-cells), are co-ordinators of the body’s immune response, e.g., providing help to B cells in the production of antibody, as well as in augmenting cellular immune response to antigens. These CD4+ T-cells are the primary target of HIV. The loss of these cells in HIV infection, both qualitatively and quantitatively, results in weakening of the immune response and the ability of the host to respond to foreign antigens, thus rendering the host susceptible to infections and leading ultimately to the acquired immune deficiency syndrome (AIDS).

Hence, the CD4+ T-cell counts are frequently monitored to assess immune suppression and disease progression in HIV infected patients. Moreover, changes in the CD4+ T-cell counts are important indicator of the response to ART5-7. At present, the CD4+ T-cell count remains the most important key surrogate marker for initiation and monitoring ART and a measure of the effectiveness of the treatment in clinical trial evaluations.

The National AIDS Control Organization has published the guideline for initiation of ART8 (Table I).

In adults the absolute numbers of CD4+ T cells are measured, while in infants and young children the relative number of these cells (percentage among total lymphocytes) is more informative. Since the CD4 counts may vary within individuals of different ethnicity, it is important to establish the reference ranges of CD4 count for the target population. The reference range for Indian healthy adult has been established in a multicentric study carried out at six different centres from different parts of India9. The CD4 percentages in Indian adults were found to be lower as compared to Western countries. The mean CD4 per cent were found to be 37 per cent (range: 14 to 65%). The CD4:CD8 ratios in southern part of India were found to be significantly lower as compared to the values from northern and western parts of India9. Various studies carried out on Indian population9-12 have showed the range of absolute CD4 counts as 600 to 1200 cells/µl.

Looking at the importance of the CD4 count estimation in the clinical setting for ART monitoring, the guidelines addressing the quality control of CD4 testing in persons with HIV infection have also been developed13-15. In this review, the methodologies of enumeration of CD4+ T-cells and various available quality control options are discussed.

### Methods for enumerating absolute numbers of CD4+ T-cells

To date, both automated and manual methods have been developed for determining the absolute number of CD4+ T-cells and evaluated in multicentric studies. These include both flow cytometric and non-flow cytometric technologies. Most of the technologies...
summarized here are commercially available as cell counting systems and/or kit packages.

**Flow cytometric (automated) methodologies:** Flow cytometry is the gold standard method for the estimation of CD4 counts due to its accuracy, precision and reproducibility and thus widely used. This technology is capable of high sample throughput and great versatility in its applications; CD4 counting is just one of its numerous uses in the biomedical field. However, flow cytometry based CD4 counting is relatively complex, and therefore technically demanding, costly and needs regular maintenance. Additionally, it is essential that operators of the flow cytometer be sufficiently trained in the technical and biological aspects of CD4 counting. Although, the system has a high initial and running cost at present, the introduction of simpler and portable instruments may change this situation in near future.

Flow cytometry provides options like dual- and single-platform approach for estimation of absolute CD4+ T-cell counts.

**Dual-platform approach:** The dual-platform approach uses two instruments to generate absolute CD4+ T-cell counts: a flow cytometer for generating a percentage CD4+ T-cells among lymphocytes and a haematological analyzer to enumerate the absolute lymphocyte counts. An absolute CD4+ T-cell count is then derived by multiplying percentage of CD4+ T-cells by the absolute lymphocyte count. Because the percentage of CD4+ T-cells is obtained from the reference lymphocyte populations, the purity of the lymphocytes is therefore needed to be defined with the greatest precision. Traditionally, in the blood sample that contains a high proportion of lymphocytes, a lymphocyte gate for CD4+ T-cell testing is easily derived from a bivariate histogram or homogeneous gate that includes forward scatter (FSC, size of the cell populations) and right angle side scatter (SSC, granularity of the cell populations) patterns. However, when there is a high proportion of non-lymphocytes (monocytes, basophils and immature red blood cells), this traditional FSC/SSC lymphocyte gate tends to be unreliable as non-lymphocytes have been shown to contaminate the gate, thus this morphological gating is now considered as unacceptable. A more reliable method for assessing lymphocyte gate purity and lymphoid cell recovery on the basis of differential CD45 marker density expression has been developed by Loken et al. This method also known as CD45 grating, which uses two markers (CD45 and CD14): CD45 is a pan-leukocyte marker expressed at different intensities on leukocytes (granulocytes CD45+; monocyte CD45++; lymphocytes CD45+++ or bright) while the CD14 marker is selectively expressed by monocytes. This CD45 gating approach can gate all lymphocytes in the acquired events and maximize their purity by excluding unwanted non-lymphocytes. Based on this CD45 gating strategy together with the advent of the flow cytometry, the more practical and accurate three-colour (CD3/CD4/CD45) or four-colour (CD3/CD4/CD8/CD45) immunophenotyping assays of CD4+ T-cells have become available. In three- or four-colour immunophenotyping techniques, lymphocytes are defined as CD45+ bright with low SSC enabling a very pure population of lymphocytes. Once this lymphocyte gate is established, the percentage of lymphocytes reactive for CD3+/CD4+ T-cells (Fig.1B) as in the three-colour assay, and/or CD3+/CD8+ T-cells as in the four-colour assay can then be easily determined.

The methodology of CD45 gating has several advantages: (i) It is easy to distinguish lymphocytes in the SSC/CD45 bright gate even in the presence of a large amount of debris, which allows the use of lyse/no-wash staining method; (ii) The isotype controls could be avoided thus reducing the cost of the reagents; and (iii) It is possible to determine % lymphocytes among leukocytes from the SSC/CD45 gate.

Recently, this CD45 gating strategy has been adopted with a more practical two-colour PanLeucogating approach. This two-colour PanLeucogating approach uses total leukocytes as the common denominator, in which total leukocytes are identified and gated by their SSC and CD45+ characteristics. CD45 is reactive with fluorochrome-conjugated antibody. Leucocytes and lymphocytes are identified and gated by drawing two regions: one around all leucocytes and the other set on all bright CD45+ cells with low SSC (Fig. 2A). Lymphocytes gated in this region are further analyzed for CD4+ T-
cells. These cells are easily distinguished from non-CD4+ T-cells and per cent CD4 is then obtained as a percentage of total lymphocytes (Fig. 2B). The same analysis protocol can also be applied to CD8+ T-cells using CD45/CD8. This CD45-assisted PanLeucogating technique is now widely accepted, since it is simple, better, and cost-effective to use in the resource-poor settings.

The dual platform approach requires the results of three separate laboratory tests: percentage CD4+ T-cells from the flow cytometer, total white blood counts and percentage of lymphocytes from the haematological analyzer. This undoubtedly leads to wide variability. The basic problems of haematological analyzer have been described in the literature\(^ {25-27}\). It is now well accepted that the use of a haematological analyzer as the second platform for white blood count and the three-or five-part differential count introduce variable factors into the calculation of absolute CD4+ T-cells. These are particularly so in the interlaboratory studies that use different haematological analyzers\(^ {28,29}\). In the individual patient follow up studies, the absolute CD4+ T-cell counts obtained from the dual-platform approach contribute to more variability than per cent CD4+ T-cell measurement (20-33% within-person coefficient variation vs. CV of 10-16%). Variation in the total white blood cell counts (CV of 16-20%) and lymphocyte differential (CV of 16-26%) over time in an individual leads to the greater variability of the absolute CD4+ T-cell counts compared with per cent CD4+ T-cells. Moreover, the patient samples contain cell numbers beyond the sensitivity and linearity range of haematological analyzer (e.g., severely leucopenic patients), the lymphocyte population defined by flow cytometer may not match exactly to that of the haematological analyzer. Further, total white blood cell counts are not subjected to regular internal quality control and external quality assurance. In spite of that, this dual-platform technology is still widely practiced and recommended in a number of institutional guidelines\(^ {13,16,17}\).

It should be noted that the dual-platform approach when combined with known density microbeads could be used as a single-platform flow cytometric method for determination of absolute number of CD4+ and CD8+ T-cells.

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**Fig.1.** Representative two-parameter dot plots of a CD3-fluorescein isothiocyanate (FITC)/CD4- phycoerythrin (PE)/CD45-peridinin chlorophyll protein complex (PerCP) stained whole blood sample by pan leucogating approach. (A) The right angle side scatter SSC/CD45 dot plot shows all leucocyte population with lymphocytes at CD45\(^ {\text{bright}}\) population (R1). (B) Identification of CD3+/CD4+ T-cells (upper right quadrant) from R1 cell population.
Single-platform approach: The single-platform approach enables absolute CD4+ T-cell counts to be derived directly without the need for a haematological analyzer. This can be assessed either by counting CD4+ T-cell populations in a precisely determined blood volume or by using the known numbers of fluorescent microbeads admixed to a known volume of CD4- stained blood. Importantly, the precision of this technology depends on the flow cytometric measurement and the methodology of pipetting in the staining process. It is very crucial that the use of reverse pipetting technique, is the most reliable dispensing method for absolute CD4+ T-cell count assay.

To date, single-platform technologies have two options of microbead-based technologies and the volumetric technologies. These alternatives are discussed below:

FACSCount™ microbead-based system: FACSCount system is the product of Becton Dickinson Biosciences. It is the only available microbead-based single-platform instrument that designed specifically for enumerating the absolute CD4+, CD8+ and CD3+ T-cell counts in no-lyse, no-wash whole blood. The system has been approved by many international organizations as one of the predicate method. This system requires ready-to-use twin-tube reagent tubes. One tube determines the absolute number of helper/inducer T-cells (CD4+/CD3+) by using a combination of two-colour monoclonal anti-human CD3 antibody conjugated to the tandem dye phycoerythrin + Cy5 (PECy5) and a monoclonal anti-human CD4 antibody conjugated to phycoerythrin (PE). The other tube determines the absolute number of cytotoxic/suppressor T-cells (CD8-+/CD3+). Both tubes also give the absolute number of total T-cells (CD3) or CD3+ T-cells, as well as CD4/CD8 ratio. In addition to the antibody reagents, the reagent tubes also contain a known number of fluorochrome-labelled reference beads. These beads function as fluorescence standard for locating the lymphocytes and also as a quantitation standard for enumerating the cells. The control set consists of fluorescent beads at four different densities: zero (0 beads/µl), low (50 beads/µl), medium (250 beads/µl), and high (1000 beads/µl).
When whole blood is added to the reagents, fluorochrome-labelled antibodies in the reagents bind specifically to lymphocyte surface antigens. After a fixative solution is added, the sample is run on the instrument. The stained cells come in contact with the green HeNe laser light, which causes the cell to fluoresce. This fluorescent light provides the information necessary for the instrument to count the cells (Fig. 3). The calculation of absolute CD3+, CD4+ and CD8+ T-cells is determined automatically by using the built-in Attractors software program.

**Guava® EasyCD4™ volumetric system:** The Guava® EasyCD4™ Reagent kit is the product of Guava Technologies. It is based on a micorcapillary cytometry technology that enables the enormous analytical and diagnostic power of conventional flow cytometry in a highly miniaturized single platform. The system contains two-colour, direct immunofluorescence reagents for enumeration of mature CD4+ T-cells in human blood. The kit consists of two monoclonal antibody directly conjugated to PECy5 and PE for CD3 and CD4 T-cell antigens. The instrument itself consists of a diode green laser. Sample acquisition is done by a variable-speed fluid pump that does not require sheath fluid and the system’s sampling precision depends on the integrity of fluid pathway. The CD3+ cells are gated and the CD3+ and CD4+ cells are identified in the gate (Fig. 4). The system showed good correlation with conventional flow cytometry and is easy to operate. It uses smaller blood volume. However, there is need for experienced technical personnel for operating the system.

**Partec CyFlow volumetric system:** CyFlow is another desktop single-platform technology made by Partec GmbH. It is a volumetric software controlled absolute count system equipped with either a single 532 nm green solid-state laser used for one fluorescence parameter or two lasers with a mercury arc lamp applicable for 2 or 3-colour analyses. Data acquisition and analysis are performed in real time with FlowMax software. The system showed good correlation with the CD4 counts obtained by conventional flow cytometry. However, experienced technician is required for accurate measurement.

**Non-flow cytometric methods:** Although flow cytometry is the accepted standard method for the determination of absolute counts of CD4+ and CD8+ T-cells, the methodology involved the use of flow cytometer and haematology analyzer, which is expensive both in term of initial investment and maintenance as well as requiring highly trained personnel. Thus it is unsuitable for routine use in most laboratories with limited facilities such as district hospitals. An ideal CD4 testing in a resource-poor setting would be an assay which is simple, uses inexpensive instrument and minimum training period that would reliably identify CD4+ T-cells without sacrificing much accuracy and precision. There are several alternative non-flow cytometric technologies available for determination of absolute CD4+ and CD8+ T-cells. These
technologies are cost-effective and thus might be suitable in the local situation in the resource-poor settings. Currently available manual assays rely on microscopes (optical or fluorescence). Additionally, they require the use of other small equipment such as centrifuges and magnets. These tests are designed to operate with low sample throughput in resource-limited laboratories but their running costs are not always lower when compared to the automated methods.

**Beckman-Coulter cytosphere system**: This manual CD4 count kit contains CD4+ cytospheres reagent, inert latex spheres coated with monoclonal antibody, used to identify and manually enumerate the absolute number of CD4+ T-cells by visible light microscopy in fresh whole blood. In this assay, 10 µl of cytosphere monocyte blocking reagent is added to 100 µl of whole blood. After incubation, 10 µl of CD4 Cytosphere reagent is added to the mixture. Ten µl of blood-latex spheres mixture is then pipetted and lysed by lysing reagent. Finally, the lysed mixture is loaded on the haemocytometer and read under the light microscope. Since the diameter of the cytospheres for CD4 reagent is different from the cytosphere monocyte-blocking reagent, CD4+ T-cells rosetted with antibody-coated latex beads can be distinguished from the monocytes rosetted latex beads due to their large latex sphere size. The Cytosphere system has advantage for those haematology technicians who are familiar with the shapes of cells. Here the monocytes are not removed but appear different under the microscope, so the bead-covered CD4+ T cells can be counted.

The Cytosphere system has been found to be useful alternative to flow cytometry for the estimation of CD4 T-lymphocyte counts, in resource-poor settings like Indian laboratories, for monitoring HIV progression and response to therapy.

**Dynabeads T4-T8 quantitative system**: This non-flow cytometric technology is the product of Dynal. The system is based on the use of immunomagnetic cell isolation method which uses Dynabeads magnetic particles coated with antibody to CD4 and CD8 antigens to capture and isolate CD4+ and CD8+ T-cells from the whole blood. In this system, the whole blood diluted with buffer solution is depleted of monocyte using monocyte depletion magnetic bead reagent coated with anti-CD14 antibody. After incubation on a Dynal rotator, magnetic separation of monocytes is done using a magnetic particle concentrator. Aliquot of the supernatant from the monocyte-depleted blood is diluted and mixed with magnetic beads coated with anti-CD4 monoclonal antibody. After incubation, the beads are separated from the CD4+ T-cells. The isolated CD4+ T-cells are then stained with gentian violet and trypan blue. The isolated stained cells are lysed and the nuclei are identified and counted manually by light microscopy or in an automated cell counter.
The method has been validated in resource poor settings in Africa\textsuperscript{39,40}. The method showed significant correlation with that obtained by flow cytometry and found to be cost effective with US$ 3/test\textsuperscript{40}.

However, these methods are manual and labour intensive. To scale these up to match-expanding access to ART may prove challenge. The automation by addition of image analysis software would increase the potential of its use in ART monitoring. The system could be cheaper than other alternatives and will be useful in small settings if it is backed up with flow cytometry for quality assurance.

**ELISA based counting systems (Capcellia ELISA and TRAx and Zymmune)**

The ELISA systems measure CD4 protein in the lysed whole blood. The system could be used in 96 well formats, thus could be automated easily and can be used on large number of samples. The Capcellia immunocapture assay has been used to estimate CD4 counts in HIV seropositives and compared with the CD4 counts obtained using flow cytometry and the plasma viral load. Capcellia for CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell counts was found to be a cost-effective, user-friendly assay, which provides counts that correlate well with HIV-1 load measurements\textsuperscript{41}. Another study done by the same group\textsuperscript{42} showed that the test can be a useful alternative for flow-based method however, the assay system has certain limitations inherent to ELISA techniques. Hence, these systems have not found favour with the investigators.

One study\textsuperscript{43} compared five alternative methods; FACSCount (uses flow cytometry), Cytospheres, Dynabeads, Opti-CIM, and Capcellia for implementation in developing countries, and concluded that FACSCount and Dynabeads methods compared well with standard flow cytometry; of these the Dynabeads method appears less expensive and potentially useful for countries with limited economic resources.

**CD4 count estimation from dried blood spots**

The technique uses an antibody ‘sandwich’ to capture and detect CD4 proteins in the sample. One study in Zambia has shown promise in the methodology showing good correlation with the conventional flow cytometry\textsuperscript{44}. However the technology is not ready for use in the field.

Table II shows details of CD4 count estimation methods available at present.

**Quality assurance in CD4 testing**

Since the use of CD4\textsuperscript{+} T-cell measurement have critical implications for the effective management of individuals at risk or infected with HIV or progressing into AIDS, it is crucial that external quality control is put in place to ensure that the test results from individual laboratories are equivalent.

The aim of quality assurance is to ensure that doctors and patients are getting the same information from the laboratory tests, no matter which laboratory they use or the methods used there.

For a good quality control, the diurnal variation in the CD4 counts should be considered as an important factor. To avoid such variation it is recommended that the sample should be collected at a specified time from an individual. Another point that has to be taken into account is the storage of sample before assessment. This period varies with the methodology used and location of the testing laboratory.

Several sets of guidelines addressing quality control of flow cytometric CD4+ testing have been developed. Though there is at present no external quality control on non-flow cytometric CD4 testing, it is important that accurate daily within laboratory (internal quality control) and proficiency test or external quality assurance programmes (EQA) be employed to ensure the reliability of CD4 data. Satisfactory performance in CD4 testing EQA is recommended for HIV research and clinical trial programmes in many parts of the world. For the most parts, internal control samples from the manufacturer are used to monitor both sample processing and instrument performance however, internal quality control practice is not without merit, it does not provide full-process quality control. There are very few laboratories, particularly in the developing world participating in international EQA programmes such as United Kingdom National External Quality
Assessment Schemes or UKNEQAS\textsuperscript{45,46}. Proper transportation of specimens and reagents is necessary for quality assessment management and availability of good quality reagent at remote places. A technology that is being developed and refined at present is a fixative that allows blood samples to be stored and transported over 5 days (or even slightly longer) without loss of accuracy in test results. The high cost of these programmes has been a major burden, making their implementation in resource-poor countries difficult. However, there are free EQA programmes such as Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS (QASI)\textsuperscript{47}, in spite of their irregular schedules; they are useful and cost saving.

In summary, the various technologies currently available for enumerating CD4+ T-cells are based on distinct principles and have different operational characteristics. New manual technologies based on micro-arrays and dipsticks are being developed and probably will become available in a few years time. It is necessary to consider the purpose of the assay (whether it is being used for monitoring or for research), the age group of the patients, sample turnover and available resources (financial, human and infrastructure) before making the choice for the test. It is also required to participate in the quality control programme to impart reliability to the results.

### References


### Table II. Available CD4 enumeration technologies

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<tr>
<th>Type of assay</th>
<th>Principle</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Flow cytometry based assays</td>
<td>PanLeucogating/ A combination of CD4 and CD45 antibodies\textsuperscript{22,24}</td>
<td>Cost effective without compromising quality</td>
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<tr>
<td></td>
<td>Volumetric method (Partec)\textsuperscript{32}</td>
<td>Cheap (2 US$ per test)</td>
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<td></td>
<td>Works on CD3+ lymphocyte gating</td>
<td>Could be useful in field</td>
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<td>Requires trained personnel</td>
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<tr>
<td>Microscope based assays</td>
<td>Uses Dynabeads to identify CD4 lymphocytes\textsuperscript{34,35,38-40}</td>
<td>Might be useful at peripheral centres</td>
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<td>Cost effective,</td>
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<td>Requires fluorescence</td>
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<td>Microscope</td>
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<td></td>
<td>Uses cytosphere for detecting CD4 cells, works on haematology principle</td>
<td>Cost-effective</td>
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<td></td>
<td>using shapes of the cells for interpretation\textsuperscript{36,37}</td>
<td>Good correlation with standard flow cytometry</td>
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<tr>
<td>ELISA based assays</td>
<td>Measurement of CD4 protein (Capcellia ELISA)\textsuperscript{41,42}</td>
<td>Detects CD4 protein in whole blood</td>
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<td></td>
<td>Measurement of CD4 protein from dried blood spot\textsuperscript{44}</td>
<td>Detects CD4 protein in whole blood</td>
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Superscript numerals represent reference numbers


