Ministry of Health and Family Welfare National AIDS Control Organisation

> NATIONAL GUIDELINES FOR THE ENUMERATION OF CD4' T-LYMPHOCYTES WITH SINGLE PLATFORM TECHNOLOGY FOR INITIATION AND MONITORING OF ART IN HIV INFECTED INDIVIDUALS

March 2007

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ACKNOWLEDGEMENTS

We are most grateful to Ms. Sujatha Rao, Addl. Secretary and Director General, National AIDS Control Organization for entrusting us with the important task of preparing guidelines for enumeration of CD4 cells, a very important component of the HIV/AIDS care and support programme.

The contributors/experts have tried to present the techniques, laboratory requirements and the needs etc. in a simple and easy to understand text. The guidelines give in detail the importance of CD4 cells; how and when to enumerate the CD4 cells; available technologies; the principles of the techniques used; basic infrastructural and training requirements; bio safety and methods of calculation of CD4 cells in adults and children. The guidelines also include the quality assurance and external quality assessment practices required for performing the test accurately and for proficiency testing of performance by laboratories.

We are extremely grateful to experts who reviewed these guidelines and provided their valuable inputs. We are indebted to Dr. J. Sokhey, Addl. Project Director NACO for the valuable guidance in compiling this document. All the suggestions made by experts have been incorporated in the guidelines.

We hope these guidelines will be used for CD4 cell estimation at sites all over India to perform the test correctly and in a standard manner to produce accurate results.

The Contributors

ABBREVIATIONS

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ART	Anti Retroviral Therapy
BD	Becton Dickinson
CDC	Centers for Disease Control
DLC	Differential Leukocyte Count
DPT	Dual Platform Technology
EDTA	Ethylene Diamine Tetra Acetic acid
EQA	External Quality Assessment
FC	Flow Cytometry
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
HBV	Hepatitis B Virus
HIV	Human Immunodeficiency Virus
IATA	International Air Transport Association
NACO	National AIDS Control Organisation
NARI	National AIDS Research Institute
NRL	National Reference Laboratory
PE	Phycoerthyrin
PLG	Panleukogating
PMT	Photomultiplier Tube
QC	Quality Control
SACS	State AIDS Control Society
SPT	Single Platform Technology
UN	United Nations
UPS	Uninterrupted Power Supply
WBC	White Blood Cells
WHO	World Health Organization

CHAPTER 1 INTRODUCTION

With a population of over 1 billion, and close to 5.21 million people infected with the Human Immunodeficiency Virus (HIV), the HIV epidemic in India will have a major impact on the overall spread of HIV in Asia, the Pacific, and around the world (1). India is second only to South Africa in terms of the overall number of people living with the disease.

Human Immunodeficiency Virus type 1 (HIV-1) infects and destroys leukocytes that express the CD4 surface receptors, as a result, depletes its host of CD4 ⁺T-lymphocytes (2, 4-9). Therefore, obtaining accurate and reliable measures of CD4⁺ T- lymphocytes is essential to assess and manage persons infected with HIV (3, 7). Depletion of CD4⁺ T-lymphocytes has been linked to the immunopathogenesis of HIV infection and the progression of the disease. From the early days on, CD4⁺ T-lymphocyte assay has been recognized as the hallmark clinical surrogate marker for staging HIV disease progression (10, 27). A CD4 ⁺T-lymphocyte count of 200 cells/µl and less in presence of HIV infection is regarded as an

AIDS defining event (11).

CD4 ⁺T-lymphocytes in HIV infected individuals are monitored for the following reasons:

- to estimate the level of immune competence of an individual
- to stage HIV disease
- to make decisions for initiation of ART.
- monitoring response to anti-retroviral therapy / to estimate rate of progression of HIV disease
- to initiate chemoprophylaxis against opportunistic infections (13-14, 26).

Scope:

These guidelines have been developed for laboratories which perform CD4⁺ T-lymphocyte immunophenotyping in persons infected with HIV. The guidelines describe single-platform technology (SPT), a process in which absolute CD4⁺ T-lymphocytes are enumerated with a single instrument. This document touches upon the various aspects of flow cytometry including, available instrument options, sample collection and transportation, laboratory biosafety, basic requirements for immunophenotyping, quality control and training needs.

CHAPTER 2

PRINCIPLES OF FLOW CYTOMETRY.

Flow cytometry is the automated analysis of optical properties of individual particles in a fluidic system. It is a process in which measurement of physical and/or chemical characteristics of cells/particles are made while cells pass through the measuring apparatus in a fluid stream. The current applications of flow cytometers are derived from their ability to define and quantify cell populations (10-12, 16).

Components of a flow cytometer (FC)

The system can be broken down into 3 basic elements:

- 1. Fluidics
- 2. Optics
- 3. Electronics

Fluidics: The flow cytometer requires the cells to be in suspension, flowing in single file through flow cell. Alignment with the laser and the detectors is extremely important if consistent light scatter and fluorescent signals from the same particle are to be obtained. To produce a good alignment, a fluid transport system is in place, starting at a test tube containing cells in suspension, from which the sample is transported to a tubing system leading to a flow chamber. Thus the liquid containing particles is injected into the centre of a second liquid stream, the sheath fluid, through an orifice of 50-300µm.

The fluidics component consists of:

- <u>Sheath fluid</u>¹: surrounds the sample fluid
- Sample inlet: where the sample is introduced to the cytometer and the flow cell
- Flow cell: where sample enters the sheath stream to be focused for illumination by the laser
- <u>Waste collection:</u> The waste is collected in the container provided with the flow cytometer.

<u>Optics</u>: A light source needs to be focused on the same point where cells have been focused by the fluidic system. As cells pass in a single file through this focus point, light and fluorescence is scattered, absorbed, excited and emitted at different wavelengths.

Flow Cytometry measures light scattered, emitted or absorbed by cells, which in turn provide the values of almost all measurable parameters. The choice of light source in cytometer is lasers. The laser beam can interact with the cell and any fluorescent marker bound to the cell surface or intracellular antigens, to scatter light and fluorescence. The most common lasers used are argon and diode lasers.

¹Sheath fluid is eliminated in the flow cytometers that make use of volumetric technology e.g. Guava and Partec Cylflow.

As cells pass through the laser beam, light is scattered in all directions. One can identify light scatter (forward scatter and side scatter) and fluorescent scatter (which relates to the fluorochrome used as antibody conjugate). Forward scatter is the amount of light scattered in the forward direction, along the axis that the laser is traveling. The intensity of forward scatter is proportional to the size, shape and homogeneity of cells or particles. Side scatter refers to the scatter of light perpendicular to the axis that the laser light is travelling and is more sensitive to inclusions within cells than forward scatter. It is also used to distinguish granulated cells from non-granulated cells

Electronics: Once the light has been collected from various directions and selected by filters, it reaches the detectors where light changes back into photons. Photomultiplier tubes (PMTs) are used to convert these photons into an electronic output. The PMT's are thus sources of electric current. The amplitudes of these pulses should be proportional to the amount of light reaching the detectors from the cell, which should be proportional to those amounts of material in the cell, which are scattering or emitting light. Pulses are converted to numbers through a process called analog-to-digital- conversion. Data acquired can be further manipulated by selecting the cell population of interest by gating. Only data within that gate will be collected and stored on the computer. Data may be displayed in a variety of ways, using dot plots, histograms or scatter plots. The quality of data acquired may be influenced by a number of aspects, like antibody concentration, gating and color compensation, etc.

CHAPTER 3 SAMPLE COLLECTION AND TRANSPORTATION

Preanalytical considerations:

- 1. Fasting conditions are not required for collection of blood specimen.
- 2. Blood specimen should be collected from the same person at similar times of the day to avoid variations in results due to diurnal fluctuations.
- 3. Use of a syringe should be avoided for drawing the sample and the sample should be collected using a vacuum based collection system. This is because use of a syringe while transferring the sample to a vacutainer tube may cause hemolysis.
- 4. For the purpose of patient follow-up, testing should be done by the same method and on same type of machine that was used for conducting the screening test.

Sample collection and handling:

- 1. Blood should be collected in K_3 or K_2 EDTA vacutainer tube (depending upon tube capacity; 3-5 ml) to a full draw.
- 2. The tube should be labeled with patient's identification, date and time of collection as well as the name of the collecting personnel. The tube labels should be double checked for accuracy with the the sample request forms before sending it to the testing lab.
- 3. The blood should be mixed properly by inverting the tube 6-8 times immediately after collection. Formation of small clots may affect accuracy of the count and ability to run the instrument.
- 4. Sample should reach the laboratory within 30 hours of phlebotomy.
- 5. Do not refrigerate or freeze the specimens. Transport and store the tubes at ambient temperature (20 -25°C). If the external temperature is high, the samples should be shipped with cool (not frozen) packs.
- 6. Samples should be processed within a maximum of 48 hours of collection.
- 7. The samples should be rejected if they are clotted, hemolysed or frozen.

Sample transport

Maintain specimens at room temperature during transportation. Avoid extremes of temperature so that specimens do not freeze or get heated above 32 °C

The following instructions are recommended for specimen transportation:

The shipment of infectious agents is regulated by the Transportation of Dangerous Goods Act and the International Air Transport Association (IATA) dangerous goods regulations (20). HIV infected specimens are classified as infectious class 6.2 substances under the United Nations (UN) no. 2814. The packaging must adhere to UN class 6.2 specifications. Packaging requires a 3 layer system as described below (see Appendix 3 for a diagrammatic representation):

- Place the tube containing the specimen in a leak-proof container (e.g. a sealed plastic bag with a zip lock or alternatively the bag may be stapled and taped) and pack this container inside a cardboard canister / box containing sufficient material (cotton gauze) to absorb all the blood, should the tube break or leak.
- ii. Cap the canister/box tightly.
- iii. Fasten the request slip securely to the outside of this canister.
- iv. For mailing, this canister/box should be placed inside another box containing the mailing label and biohazard sign.

The diagram in appendix 3 depicts the method of CD4 sample transport for a single/ few (2-3) samples that could fit into the secondary container shown in the diagram. The size of the primary sample container will vary with the number of samples being transported. For a larger number of samples ,a tube rack (or some such container) may be used wherein the samples can be transported in the upright position and at appropriate temperature. The packaging instructions for the transport of a larger number of samples are given below:

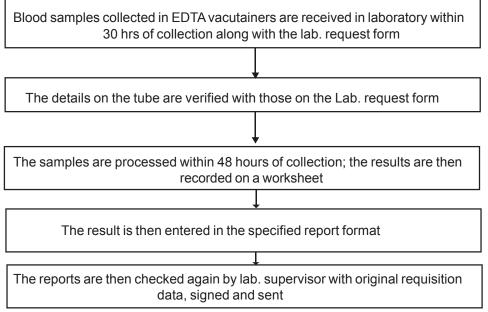
- The specimen should be carefully packaged to protect it from breakage and insulated from extreme temperature
- Label for "*CD4 count*". The collection site should make use of a unique identification number as sample identity. Names of the patients should be avoided to prevent confusion on account of duplication of names as well as to maintain confidentiality.
- Secure the vacutainer cap carefully and seal it further with sticking tape (placed so that it covers the lower part of the cap and some part of the tube stem).
- During packaging, the tubes containing specimens should be placed in a tube rack and packed inside a cool box (plastic or thermocol) with cool gel packs (so that the temperature during transport

is maintained at 20-25°C) placed below and on the sides of the tube rack. Place some cotton or other packaging material between the tubes to ensure that they do not move or rattle while in transit. Cool box required for transportation could be a plastic bread box or a vaccine carrier. Seal/ secure the lid of the cool box .

- This cool box should then be placed in a secure transport bag for purposes of shipping to the testing facility. The request slips should be placed in a plastic zip lock bag and fastened securely to the outside of the cool box with a rubber band and sticking tape.
- A biohazard label should be pasted on the visible outer surface of the package containing the samples. The package must be marked with arrows indicating the 'up' and 'down'side of the package
- Samples should be transported to the receiving laboratory by commercial courier or be hand delivered by a trained delivery person.
- The collection site must have prior knowledge of the designated testing days of the laboratory to which the samples are being sent.
- No transport should be done during weekends and holidays or non-testing days of the testing laboratory unless prior arrangement has been made with the receiving laboratory.

Note : Use overnight carriers with an established record of consistent overnight delivery to ensure arrival of specimen within the specified time. Please refer to "Guidelines for Collection, Handling and Transport of specimens for CD4 testing" for further details.

Chain of custody from sample collection to result reporting



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CHAPTER 4 TECHNIQUES FOR ENUMERATION OF CD4⁺ T- LYMPHOCYTES

Flow cytometry based methods are currently the standard technology for CD4 T⁺-lymphocyte counting because they are accurate, precise, fast and reproducible. This can be performed either by dual or single platform technology (DPT / SPT). The DPT requires a flow cytometer to generate the percentage of lymphocytes that are CD4-positive, and a hematology analyzer to obtain the total white blood and lymphocyte counts (15). The DPT is not recommended in this guideline as with this technique there are two sources of instrumental error and the hematology instrument requires blood that is <6 hours old. With SPT, only a flow cytometer is required to provide the results (16-18). See table 1 below.

There is currently one type of kit available for manual microscopic CD4 T-cell enumeration i.e. the Dynal system, which can work with either light or epi-fluorescent microscope (15, 25). This method may be practical for remote locations where the installation of a flow cytometer is not possible and the sample volume is very low.

Operating principle	Description of processing	Model name & manufacturer	% CD4	Approvals
Cytometry based methods				
Flow	Dedicated flow rate with beads	FACScount, (Becton Dickinson)	No	FDA
Flow	Flow rate with beads	FACScalibur(Becton Dickinson)	Yes	FDA
Flow	Flow rate with beads	EPICS XL(Beckman Coulter)	Yes	FDA
Flow (µ capillary)	Dedicated volumetric	*Guava easy CD4(Guava)	Yes	FDA
Flow	Universal volumetric	*Cyflow Counter (Partec)	Yes	CE

Table 1. Methodologies for CD4 ⁺ T- lymphocyte enumeration (single platform)

* Separate Kits required for % CD4 count.

CHAPTER 5 LABORATORY BIOSAFETY

In a clinical immunology laboratory environment HIV can be accidentally transmitted either by percutaneous (e.g. through a needle stick) or mucous membrane exposure by splashing of infectious fluids. Another route of infection is the exposure of non-intact skin with infectious fluids in which case the virus may invade and inoculate the worker through minor cuts, scratches, abrasions, and skin lesions. Aerosol transmission of HIV in a flow cytometry laboratory has been hypothesized, but not documented. It is thus important that the operators of flow cytometers be protected from exposure to accidental pricks, contaminated surfaces, accidental splashes, droplets and aerosols (10).

All laboratories are required to adhere to the standard work precautions (20-23).

The following general instructions should be followed in each laboratory.

- 1. Take protective measures to avoid direct physical contact with blood such as gloves, gowns, plastic aprons and foot wear.
- 2. Cover open cuts or wounds with waterproof dressing before work.
- 3. Gowns should be closed in front, (up to the neck and knees) and with the cuffed sleeves.
- 4. Do not wear sandals or open style shoes.
- 5. Wash hands regularly with water and soap before and after handling blood samples. If bar soap is used, it should be dry. In case of liquid soap, the container should be cleaned and maintained regularly.
- 6. Do not interchange micropipettes, multi-channels and other equipments between the labs
- 7. All the staff should be immunized against hepatitis B.
- 8. Do not eat, drink, smoke, handle contact lenses or apply cosmetics in the laboratory.
- 9. Store food outside the work area in cabinets or refrigerators designated for this purpose only.
- 10. Prior to consumption of any food, remove potentially contaminated protective clothing, wash hands thoroughly and exit the work area.
- 11. Do not leave the lab doors open.
- 12. Use and care of gloves:
 - a. Use gloves when prolonged contact with blood/body fluid is expected.
 - Rings or hand jewellery which may interfere with glove functioning should be removed before wearing gloves.
 - Gloves should be discarded (in the appropriate container with bleach) immediately after
 use and prior to contact with the environment outside the immediate work area.
 - d. Do not leave the lab with gloves on.

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- e. Do not touch any instrument/equipment in the lab with gloved hands.
- f. Do not touch eyes, nose, mouth or any uncovered body part with gloves.
- g. Do not touch telephone receiver, door handles with the gloved hands.
- h. Do not touch tap with gloved hand. Open it with elbow. Dispose off gloves in the appropriate container labeled with biohazard sign.
- 13. Avoid use of sharps and glass vials etc. wherever possible. Follow safe procedures for handling and disposal of sharps.
 - a. Do not recap/shear or bend used needles.
 - b. Never pick the sharp items with gloved hands alone. Use forceps to lift the sharps.
 - c. Dispose off sharps in puncture proof container filled with freshly prepared 1% hypochlorite solution.
- 14. Management of spillage:
 - a. Put adsorbent material on the spillage area after wearing gloves.
 - b. Pour freshly prepared 10% hypochlorite solution upon and around it.
 - c. Allow 30 minutes for the disinfectant to work.
 - d. Place the adsorbent material in the biohazard bag meant for infectious waste.
 - e. Reapply the disinfectant solution to all exposed surfaces and mop up.
- 15. Working in Biosafety cabinets:
 - a. Keep one bottle with 70% ethanol and one with 10% bleach ready for each laminar flow.
 - Keep a container with hypochlorite solution for discarding contaminated pipettes and tips.
 Prepare fresh bleach solution every day for the discard container.
 - c. Do not keep anything onto the flow gates to ensure proper flow and to maintain sterility in the hood.
 - d. Before and after working in the hood, work surface should be wiped down with 70% ethanol, the sash should be closed, the fans turned off and the UV light turned on for at least 15 minutes.
 - e. Do not overload the cabinet with unnecessary materials as they may interfere with airflow.
 - f. After switching on the motor wait for about 30 sec to allow the airflow to settle down and then check the airflow indicator to ensure that it is safe to proceed.
 - g. Work with hands well inside the cabinet and avoid moving arms in and out as contaminated air may be pumped out with them.
 - h. Swab the working surfaces and walls with disinfectant for daily decontamination.

 For thorough decontamination, which is required after large spillages, before maintenance, filter changing and testing, fumigation is necessary.

Procedure of fumigation:

Formalin (60ml/cm³ cabinet volume) + potassium permanganate are placed inside the container in the cabinet. The front closure is put in place and sealed if necessary. The cabinet is left closed overnight. The next morning the cabinet fan is switched on and front closure is opened slightly to allow air to pass in and purge the cabinet of formaldehyde. After several minutes front closure is removed and the cabinet fan is allowed to run for 30 minutes. Any obvious moisture remaining on the walls and floor can then be wiped away.

16. Working with pipettes (for details see appendix 2):

- a. Get trained in using pipetting devices used in the laboratory.
- b. Do not blow out the last drop in the pipette.
- c. Avoid mouth pipetting and bubbling while mixing the fluids.
- d. Use plastic instead of glass Pasteur pipettes for hazardous materials.
- e. Discard the pipettes in discarding jar in such a way that the rim of discarding jar is not contaminated and pipettes are completely submerged in disinfectant solution.
- f. Calibrate the micropipettes every three months and document the calibration
- 17. Discard jars:
 - a. The jars should be robust and autoclavable. The most serviceable articles are 1L polypropylene jars.
 - b. Fill 1L jars with 750 ml of diluted disinfectant (1% hypochlorite).
 - c. Replace the bleach solution in the discard jars once daily even when they have received little material during the time.
- 18. Opening of the sample tubes:

Take care while opening the tube. In the conditions where large film of liquid is likely to be trapped between the tube and the closure, open the container by gripping the closures through a strip of paper which is wrapped around both the closure and the top of the container. Paper and the cap may then be discarded into disinfectant, and if necessary, a fresh cap used.

19. Occupational Exposure and Post-Exposure Prophylaxis

An "exposure" that may place a Health Care Provider (HCP) at risk of bloodborne infection is defined as a percutaneous injury (e.g. needle-stick or cut with a sharp instrument), contact with the mucous membranes of the eye or mouth, contact with non-intact skin (particularly when the exposed skin is chapped, abraded, or afflicted with dermatitis), or contact with intact skin when the duration of contact is prolonged (e.g. several minutes or more) with blood or other potentially infectious body fluids.

Body fluids that are potentially infectious include - blood, semen, vaginal secretions, cerebrospinal fluid, synovial, pleural, peritoneal, pericardial and amniotic fluids or other body fluids contaminated with visible blood. Exposure to tears, sweat, urine, faeces, saliva of an infected person is normally not considered as an "exposure" unless these secretions contain visible blood.

Management of Exposure :

Steps to be taken on accidental exposure to blood (or body fluid containing blood) are:

- Wash wound immediately with running water and soap
- Inform the lab /hospital management and document occupational accident
- Consult with nearest ART centre/ resource for Post-exposure prophylaxis, evaluation, and follow-up (as per the National guidelines on PEP)
- Counselling and collection of blood for testing from the exposed HCW with written informed consent must be done.
- Whenever possible confidential counselling and testing of source for Hepatitis, HIV etc must be done. A history should be taken as well to ascertain likely risk of the source. (PEP should be provided to the exposed HCW until report of source is available and confirmed negative.).
- Risk of infection and transmission must be evaluated
- Never delay start of therapy due to debate over regimen. Begin with basic 2-drug regimen, and change if warranted, once expert advice is obtained
- Reevaluation of the exposed person should be considered within 72 hours post exposure, especially as additional information about the exposure or source person becomes available. The exposed person is advised to seek medical evaluation for any febrile illness that occurs within 12 weeks of exposure.
- Administer PEP for 4 weeks .PEP should be provided until result of the source's test is available and confirmed negative or until course completed ,if source positive or unknown.
- A repeat HIV test of the exposed individual should be performed at 6 weeks, 12 weeks and 6 months post-exposure, regardless of whether or not PEP was taken

Ideally, prophylaxis should be begun within 2 hours of exposure.

Donts:

- Do not panic!
- Do not reflexively place pricked finger into mouth
- Do not squeeze blood from wound, this causes trauma and inflammation, increasing risk of transmission
- Do not use bleach, alcohol, betadine, or iodine, which may be caustic, also causing trauma

Dos:

- Remove gloves, if appropriate
- Wash site thoroughly with running water. Irrigate thoroughly with water or saline if splashes have gone into the eye or mouth.

CHAPTER 6

QUALITY MANAGEMENT

CD4+ T lymphocyte count is often the only available tool for disease progression monitoring during ART, therefore maintaining consistent and reliable test performance is important to maintain quality patient management. Quality control is associated with the reduction of error. It is defined as the systematic procedure in the laboratory to evaluate and monitor the accuracy and precision of the CD4⁺ T-lymphocyte measurement. Quality control includes monitoring both between (inter) and within (intra) laboratory performance. It is to assure the day to day consistency of CD4⁺ T-lymphocyte enumeration

Internal quality control for CD4+ T-cell enumeration

Some general quality control principles applicable to flow cytometres are as under:

- The methods and protocols should be available in the form of SOPs.
- Reagents must be stored as recommended by the manufacturer and daily temperature logs should be maintained.

All reagents used must be logged onto log sheets with the date opened, expiry date and signature. Reagents discarded due to contamination, spillage, etc. must be logged in the appropriate log sheets.

- Negative and positive control for CD4⁺ T-cell enumeration supplied by the manufacturer should be used. All QC results should be entered onto Levy-Jennings plots to monitor any trends, shifts or bias in results.
- All quality control failures must be logged and corrective action completed before sample analysis takes place.
- Tube to tube variation in CD4⁺ T-cell enumeration can be monitored by inclusion of same antibody cocktail in separate tubes within the one patient test series.
- Light scatter patterns may be examined for each tube within the panel for variation from tube to tube. Similarly, the number of gated events and / or time to acquire events should not vary greatly from tube to tube.
- Other potential sources of error, which need to be checked may include inappropriate gating leading to exclusion of relevant cells, tubes in a panel being run in the wrong order, inappropriate cut-offs between negative and positive cells and calculation or transcription errors.
- <u>Disinfecting the flow cytometer</u>: Disinfect the flow cytometer as recommended by the manufacturer. One method is to flush the flow cytometer fluidic chambers with a 10% sodium

hypochlorite solution for 5–10 minutes at the end of the day and then flush with water or saline for at least 10 minutes to remove excess bleach, which is corrosive (10).

- Check the fluidics system for debris, proteins and salt crystals. Identify and clean obstructions at the sample injection port and the tubing of the fluidics system (follow manufacturer's instructions)
- Pipettes must be calibrated at regular intervals as pipetting is crucial for precise enumeration of CD4⁺ T-lymphocytes.
- Maintain all equipment maintenance and calibration records

External Quality Assessment for CD4+ T-cell enumeration

External quality assessment is widely recognized for quality laboratory testing. A well functioning national programme is an important step towards achieving high quality laboratory performance on a national scale. External quality assessment (EQA) is evaluation by an outside agency of the performance by a number of laboratories on specially supplied samples. Analysis of performance is retrospective. The objective is to achieve between-laboratory and between-method comparability.

Objectives of EQA

- The primary objective is to continually improve and maintain the high standard of the laboratory's performance.
- To continually institute a formal monitoring and evaluation programme.
- To continually promote the concept of quality assurance, quality control, and quality assessment in the laboratory.
- To assess the quality of service of the participating laboratory.
- To identify problems and take appropriate interventions for corrective actions.
- To encourage the implementation of good laboratory practices.

External quality assessment (EQA) is an important complement to internal quality control. Even when all precautions are taken to achieve accuracy and precision in the laboratory, errors arise which are only detectable by objective external assessment. The principle is that the same material is sent from an international, national or regional centre to a large number of laboratories. It is important that surveys should be performed at regular intervals.

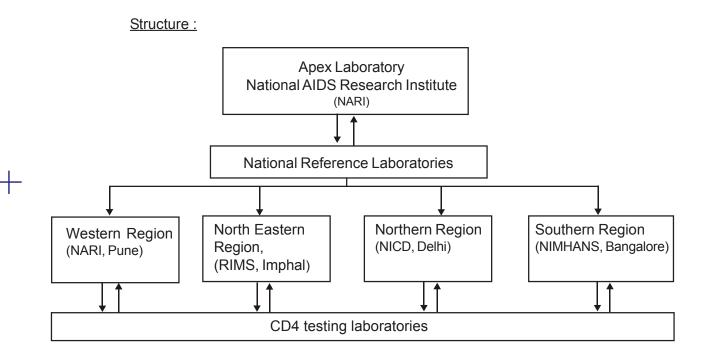
The EQA essentially contains the following components:

- 1. Filling up questionnaire to understand the laboratory capabilities and additional requirements
- 2. Training for CD4 count estimation procedure and participation in EQAS

- 3. Processing of the samples received under EQAS by the routine procedure for CD4 count estimation
- 4. Submission of report to the conducting laboratory
- 5. Identifying reasons for discordance of results and trouble shooting.
- 6. Analysis of data and reporting it to the apex lab.

Implementation of CD4 EQA Program

<u>Frequency</u> : The EQA has to be conducted at regular intervals.



Roles and responsibilities

- <u>Apex Laboratory:</u> The apex laboratory will be responsible for EQAS working modalities for distribution of samples to all the participating centers and NRLs, analysis of the results received from the participating centers, give feedback to the participating centers and trouble shooting².
- <u>National Reference Laboratories (NRLs</u>): The NRLS will be responsible for collating and sending the reports of participating labs in their territory to the apex laboratory and help the participating labs in trouble shooting and modalities of EQAS.
- Participating laboratories: The participating laboratories will be responsible for filling up the questionnaire, processing of EQAS samples, documentation and sending the reports to NRLs. The laboratories should take initiative in contacting NRLs, SACS and apex

² The apex lab shall also be responsible for the preparation and further distribution of test panels to the NRLs as and when the technology transfer takes place

laboratories for trouble shooting and problems encountered during CD4 count estimation.

Procedure:

- Each participating laboratory will be issued one code number to keep the confidentiality. The stabilized blood samples will be sent to participating laboratories by apex laboratory with the reporting formats for EQA.
- 2. The participant laboratories would process the EQAS samples as routine samples and send the reports in the prescribed formats to their respective NRLs.
- 3. The NRLs would send reports from all the participating laboratories affiliated to them to apex laboratory with a copy to The Project Director, State AIDS Control Society of respective state within 5 days from the receipt of the sample.
- 4. The apex laboratory would compile the results and send them to the organizing agency. The apex laboratory will give feedback on results of all the laboratories by giving only code number and highlighting results of respective lab. This would enable assessment of all the results without disclosing the identity of the laboratories and allow provision of taking corrective action as and when required.
- 5. The apex laboratory would provide trouble shooting support to the participating laboratories with the help of NRLs. In case of any default at any centre, the respective NRLs will troubleshoot through training in elements of quality control and may visit the centre if required for improving the performance.

The National AIDS Control Organization (NACO) will facilitate the conduct of EQAS for CD4 testing by sending advisories to the Project Directors, State AIDS Control Societies, Dean of the College / Institute, Head of the Deptt. of Microbiology and the Officer-in-charges of CD4 laboratories and by strong advocacy for this activity. NACO will also provide the necessary financial support for the conduct of EQAS.

CHAPTER 7 BASIC LABORATORY REQUIREMENTS.

This section enlists the minimum infrastructural requirements in a CD4 testing laboratory and an ideal laboratory floor plan.

Electrical requirements

- (i) Two 220 VAC, 50/60 Hz (± 10%), 20-amp dedicated circuits.
- (ii) Stable electricity supply or dedicated back-up generator if power cuts are regular

NOTES:

- (i) The cytometer/loader requires one circuit while other laboratory equipment (e.g. the computer, fridge) requires a separate circuit.
- (ii) Uninterrupted power supply (UPS) unit required
- (iii) A dedicated circuit is defined as not shared from the electrical source with any other equipment.
 Operating other equipment on the same electrical circuit may cause intermittent failures resulting in loss of data or component failures.

Operating Environment

- A normally filtered, air-conditioned environment with the ambient temperature maintained between 16°C and 29°C is required. A split unit air-conditioner of adequate rating should be installed at an elevated position well above the benchtop.
- (ii) The flow cytometer instrument and accessories dissipates up to 6500 BTU/h
- (iii) The fridge and computer will also dissipate heat, hence minimum 12,000 BTU/h required
- (iv) 2-5 laboratory personnel will work in the room
- (v) Environment with excessive mechanical vibrations should be avoided for optimum performance of the instrument. Avoid selecting sites near heavy-duty mechanical equipment as floor vibrations may impact the performance of the instrument.

Laboratory bench

- A stable, well supported and level worktable or counter space made of granite or other cleanable acid resistant laboratory surface is required.
- (ii) <u>Width:</u> minimum 300 cm
- (iii) <u>Depth:</u> minimum 80-90 cm
- (iv) <u>Height above floor:</u> minimum 80-100 cm
- (v) <u>Clearance above bench:</u> minimum 150 cm

Walls and floor

- (i) Tiles for 1.0 1.5 meters above benchtop
- (ii) Washable gloss, semi-gloss or equivalent paint on walls
- (iii) Washable and acid resistant tiles on floor

<u>Sinks</u>

- (i) A laboratory sink should be made available stable in the CD4 testing lab.
- (ii) A hand washing sink should be available in the testing lab or in adjacent rooms before exiting the overall laboratory area.

Lighting

Adequate fluorescent tube laboratory lighting should be provided

Storage

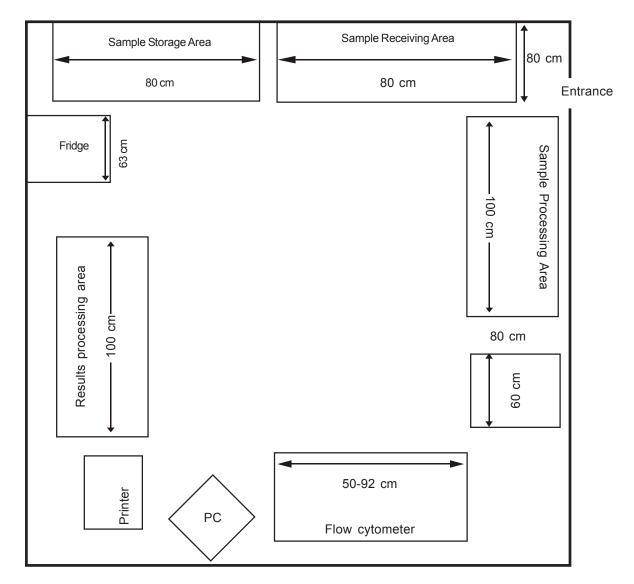
- (i) <u>Cold reagent storage:</u> Laboratory refrigerator with minimum 250-300 liter capacity. Freezer storage compartment not required.
- (ii) <u>Consumable storage:</u> Laboratory cupboards at room temperature
- (iii) <u>Results archive:</u> Filing cabinet

Waste disposal

- (i) Minimum 3 floor standing waste disposal bins with lids are required.
- (ii) Bench-top discard jars/bins are required.

Other Lab equipment / consummables

- i. Variable auto pipettors. ($20-200 \ \mu l$ and $100-1000 \ \mu l$)
- ii. Pipette tips.
- iii. Vortex / cyclomixer.
- iv. Blood rocker
- v. Plastic tube stands.
- vi. K_2/K_3 EDTA vacutainers.
- vii. Sodium hypochlorite.
- viii. Needle destroyer.
- ix Disposable gloves (powder less)



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CD4 laboratory floor plan

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CHAPTER 8 STANDARD OPERATING PROCEDURES

This section includes the standard operating procedures for the various available options / technologies in CD4 immunophenotyping, described in brief. For detailed procedures the laboratory should refer to the respective manufacturer's manuals.

BD FACSCount System

Principle

The BD FACSCount system is designed to use unlysed whole blood, collected in EDTA. When whole blood is added to the tubes of a sample reagent pair, the fluorochrome-labeled antibodies bind specifically to antigens on the surface of lymphocytes. The BD FACSCount system employs a two-colour immunofluorescence method for enumerating absolute lymphocyte counts (cells/µl whole blood) of CD3+ T-lymphocytes, CD3+ CD4+T-lymphocytes and CD3+CD8+ T-lymphocytes. CD3 cells fluoresce red and CD4 and CD8 cells fluoresce yellow when analysed on the instrument. In addition, the CD4:CD8 ratio is provided. A known number of reference beads is contained in each reagent tube and functions as fluorescence and quantitation standard for calculating the absolute counts T-lymphocytes.

- a. Reagents and controls should be kept at 2–8°C or at specified temperature until expiration date on kit.
- b. Store blood samples at room temperature and away from direct sunlight. Fresh EDTA whole blood samples are stable for up to 48 hours prior to testing.
- c. Processed blood samples can be stored at room temperature away from sunlight and remain stable for a maximum of 48 hours after the addition of the fixative solution.
- d. Processed control samples can be stored at room temperature away from sunlight and remain stable for a maximum of 72 hours after the addition of the fixative solution but only 2 hours after the addition of the control beads as per the BD FACS Count guidelines.

Reagents and Procedure for Double Reagent Pair

Reagents

- Unit test reagent pairs
 - o Tube of CD4/CD3 reagents and reference beads
 - \circ $\;$ Tube of CD8/CD3 reagents and reference beads $\;$
- Fixative solution (5% formaldehyde)
- Sheath fluid

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Procedure

- Vortex reagent tubes for 5 seconds.
- Pipette 50µl of whole blood into each tube of the reagent pair and vortex.
- Incubate the tubes at room temperature for 60 120 minutes in dark.
- Add 50µl of fixative solution into each tube and vortex.
- Run the tubes on the instrument.

Note: Stained samples may be stored for up to 24 hours before running on the flow cytometer at temperature 20 to 25 $^{\circ}$ C

Reagents and Procedure for Single Reagent Tube (CD3/CD4 only)

Reagents

- Unit test reagent tube
 - o Tube of CD4/CD3 reagents and reference beads
- Fixative solution (5% formaldehyde)
- Sheath fluid

Procedure

- Vortex reagent tubes for 5 seconds.
- Pipette 50µl of whole blood into the reagent tube and vortex.
- Incubate the tubes at room temperature for 60 120 minutes in dark.
- Add 50µl of fixative solution into each tube and vortex.
- Run the tubes on the instrument.

Note: Stained samples may be stored for up to 24 hours before running on the flow cytometer at temperature 20 to 25 ° C.

The BD FACScount does not generate %CD4 counts. However, the same can be calculated by using the following formula:

% CD4 count = Absolute CD4 *T-lymphocyte count Total lymphocyte count Absolute CD4 *T-lymphocyte count : as obtained by the BD FACScount

Total lymphocyte count can be obtained by a cell counter or alternatively obtained using the following formula

Total lymphocyte count = Total no. of lymphocytes(DLC) x Total leukocyte count100

BD FACSCalibur

Principle

BD FACSCalibur is a flow cytometer which is capable of measuring the scatter and the fluorescence parameter. It can detect the scatter parameter namely the forward and the side scatter which gives information about the size and granularity of the cell. The BD FACSCalibur can detect up to 3 fluorescence parameters. It can measure both absolute CD4 ⁺ T-lymphocyte count as well as % CD4 count.

Recommended antibody panels

BD TriTEST[™] CD3 fluorescein isothiocyanate (FITC)/CD4 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP) is a three-color direct immunofluorescence reagent to identify and determine the percentages and absolute counts of mature human T lymphocytes (CD3) and helper/inducer (CD3⁺CD4⁺) T-lymphocyte subsets in erythrocyte-lysed whole blood. When used with TruCOUNT[™] Tubes, absolute counts of these populations can be enumerated from a single tube.

Procedure

- Take a trucount tube.
- Add 20 µl of tritest antibody.
- Add 50 µl of well mixed whole blood collected in K₂ EDTA and vortex
- Incubate in dark for 15 minutes at RT.
- Add 450 µl of 1X lysing solution and vortex.
- Incubate in dark for 15 minutes
- Acquire on the BD FACSCalibur.

Other considerations

The instrument has to be kept on for 15 minutes before processing the samples on the BD FACSCalibur

• Lyse no wash calibration has to be selected while performing the calibration. Check that the instrument is kept in the pressurized mode and that the sheath fluid is there in the sheath tank and the waste tank is empty. Add 400 ml of sodium hypochlorite in the waste tank so that the waste does not require any pretreatment before the discard.

Guava Easy CD4 system

Principle

The Guava EasyCD4 assay allows enumeration of CD4⁺ or CD8⁺ lymphocytes in human peripheral blood using two fluorescence parameters in combination with forward scatter(FSC). Whole blood is

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incubated with a combination of anti-CD3 and anti-CD4 monoconal antibodies. The anti-CD3 is conjugated to a fluorochrome called PE-Cy5 and the anti-CD4 is conjugated to a fluorochrome called PE. CD3 is an antigen that is specific for T lymphocytes and is used in this assay to identify the T-lymphocyte population. CD4 is expressed on a subpopulation of T lymphocytes, the T helper cells, as well as on some of the monocytes in peripheral blood. The assay identifies the CD4⁺ T - cells as those cells that simultaneously express both CD3 and CD4. This allows the CD4 monocytes, which do not express CD3, to be excluded from the absolute count of the CD4⁺ T cells. The data is acquired on the computer and analysed using the software. The results are reported as the absolute number of CD4 T cells per micro liter of blood. It can also determine % CD4 count.

Reagents

- Anti-Human CD4- PE
- Anti-Human CD3 PE Cy 5
- Guava 1X Lysing Solution
- Guava Check Guava Instrument Cleaning Fluid
- Deionized, distilled or RO water
- 10% Bleach Solution

General considerations

- Venous blood specimen (4.0-5.0 ml) into a K₃ or K₂ EDTA blood collection tube.
- Blood should be stained within 48 hours of collection for optimal results.
- Unstained anticoagulated blood should be maintained at 20 to 25°C prior to sample processing.
- The total preparation time for one sample is about 30 minutes and the acquisition time is about 2 to 3 minutes.
- Samples should be analyzed within 5 hours of staining.

Procedure

- 1. All reagents should be brought to room temperature prior to use.
- 2. Working solution of Guava Easy CD4 and Guava Lyse should be prepared daily.
- 3. Prepare a cocktail of 1µl each of CD4 and CD3 antibodies in 9ml of dilution buffer and vortex.
- 4. Add 10µl of whole blood to the above 10µl cocktail of the antibody and vortex for 2-3 seconds.
- 5. Incubate for 15 min in dark at room temperature (20-25°C).
- 6. Add 180ml of 1X lysing solution to bring total volume to 200µl and vortex for 2-3 seconds.
- 7. Incubate in dark at room temperature for 15 min.
- 8. Vortex and acquire the sample on the instrument.

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Note

- The Guava Check kit is used to verify the performance of Guava PCA system by assessing counting accuracy and fluorescence detection using a standardized fluorescent bead reagent. The software displays the %CV and the averages for the particles /ml (bead count), FSC intensity, PM1 and PM2 mean fluorescence (MFI) have to be monitored by the operator.
- 2. Optical alignment is not required to be checked.

Panleukogating (PLG)

This is another technique for flow Cytometry which can be performed on single platform flowcytometers such as the FACScalibur and Epics XL (Beckman Coulter).

Principle

Fluorochrome labelled CD4 and CD45 monoclonal antibodies are added to the whole blood sample. When analysed on the flow cytometer, cells that have bound the labelled antibody can be identified on the basis of their specific fluorescence emission related to the specific fluorochrome attached to either the CD45 or the CD4 antibody. The CD4 positive lymphocytes are further resolved from the rest of the white

blood cells, specifically monocytes, on the basis of their complexity (side scatter), their specific CD45 and CD4 expression.

General considerations

- 1. Venous blood specimen (3.0-5.0 ml) into a K_3 or K_2 EDTA blood collection tube.
- 2. The sample is stable for up to 5 days under suitable conditions, i.e. away from direct sunlight, not exposed to extreme temperatures or dramatic temperature changes, etc.
- 3. Acid Citrate dextrose is an acceptable anticoagulant, but not a preferred one.
- 4. Heparin samples should NOT BE PROCESSED as white cells clump when exposed to heparin, and this affects the white blood cell count.
- 5. Do not prepare samples which contain less than 2ml of blood.

Note:

Whole blood samples are stored and prepared and at room temperature, until analysis. However, if they are to be stored for longer than 2 hours before analysis, they should be kept at refrigerated at 4-7°C.

Reagents:

- Immunoprep kit
- CD45-FITC/CD4-PE monoclonal antibody
- Isoflow sheath fluid

Flow Count Beads for laboratories performing single platform CD4Flow Check <u>Procedure</u>

- 1. Place the blood samples on the blood mixer to ensure adequate mixing of the blood before pipetting.
- Pipette 10µl of the PLG CD4 monoclonal antibodies (CD4 and CD45) into the bottom of each labelled sample tube.
- 3. Add 100µl of well mixed, whole blood to the sample tube containing the monoclonal antibodies.
- 4. Vortex each tube gently for a few seconds.
- 5. Incubate the samples for a total of 20 minutes at 20-25 °C.
- 6. Add 100ml of Flow count beads to each sample. Analyse immediately or within a maximum of 2 hours after the beads have been added.

TROUBLESHOOTING

While conducting the CD4 T⁺ lymphocyte enumeration, there will be occasions when things may go wrong. A problem can occur because of instrument, reagent or technician related error. Staff should be trained to recognize when there is a problem and how to correct it. In all such cases manufacturer's instructions for troubleshooting should be followed. These are listed in the respective instrument manuals.

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ANNEXURE

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APPENDIX 1

Factors affecting CD4⁺ T-lymphocyte counts

There are certain other factors including some physiological states that may adversely affect CD4⁺ T-lymphocyte counts. These include:

Transient reductions	•	Diurnal variations in normal donors
	•	Physiological stress (post-operative,
		immediately after exericse ²⁸)
	•	Exogenous steroids, cytotoxic drugs,
	•	Intercurrent infections (urinary tract and
		influenza),
Transient increases	•	Women in follicular phases of menstrual cycle ²⁹
Long term reductions	•	Systemic Lupus Erythematosus ³⁰
	•	Sarcoidosis (margination into tissues)
	•	Primary immunodeficiencies
Long term increases	•	Smoking ²⁷

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ANNEXURE

APPENDIX 2

PIPETTING TECHNIQUE

Measurement of absolute CD4 counts requires accurate and precise measurement of blood and beads. Reverse pipetting technique is recommended for dispensing these products.

Procedures

The following information is consolidated from operational instruction manuals from several pipette manufacturers. Complete information and more detailed instructions may be seen in the specific pipette instruction manuals. Read the manufacturer's manual should carefully be read before beginning the pipetting procedure.

- Choose the appropriate disposable pipette tips for your application.
- Hold the pipette in one hand and use a slight twisting movement to seat the tip firmly on the shaft of the pipette to ensure an air-tight seal

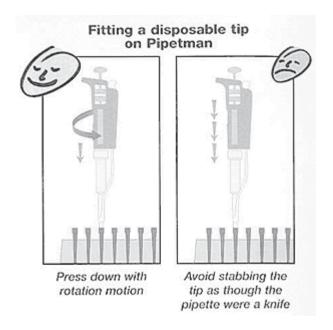


Figure 1

- Select the desired volume (with manual pipettes, higher volumes should be set first; if adjusting
 from a lower to a higher volume, first surpass the desired volume and then slowly decrease the
 volume until the required setting is reached).
- If applicable, select the desired mode (e.g., reverse pipette). This is recommended for optimal precision and reproducibility.
- Reverse pipetting can be done with a manual pipette by pressing the control button slightly past the first stop when aspirating, taking up more liquid than will be dispensed, then pressing the control button only to the first stop when dispensing. A small volume will remain in the tip after dispensing.
- Select an appropriate tip.

Pre-rinsing

The following procedures will help ensure optimal precision and accuracy.

<u>Volumes >10 µL</u>: Prerinse pipette 2–3 times for each new tip (this involves aspirating and dispensing liquid several times). Reasons for prerinsing include the following:

- to compensate for system pressure, for slight differences in temperature between pipette and liquid, and for proper-ties of the liquid;
- to clear the thin film formed by the liquid on the inside of the pipette. Without prerinsing, retention
 of a thin film on the inside wall of the tip would cause the first volume to be too small. The thickness
 and nature of this film, and therefore the potential source of error, will vary depending on the
 nature of the liquid being pipetted.

<u>Volumes <10 μ L</u>: Do not prerinse pipette, but rinse tip after dispensing to ensure that the whole volume was dispensed. For smaller volumes, prerinsing is not recommended because the dispensed volume may be higher than the requisite volume.

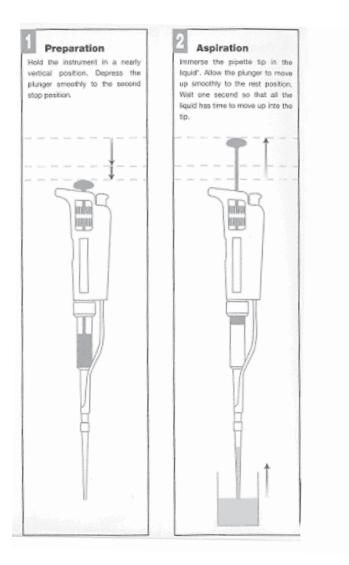
Filling (see figure 2 below)

- Make sure tip is securely attached.
- Hold pipette upright.
- When aspirating, try to keep the tip at a constant depth below the surface of the liquid.
- Glide control button slowly and smoothly (electronic pipettes perform this step automatically).
- When pipetting viscous liquids (e.g., whole blood), leave the tip in the liquid for 1–2 seconds after aspirating before with drawing it.
- After liquid is in the tip, never lay the pipette on its side.

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Dispensing

- Hold the tip at an angle, against the inside wall of the vessel/ tube if possible.
- Glide control button slowly and smoothly (electronic pipettes perform this step automatically).





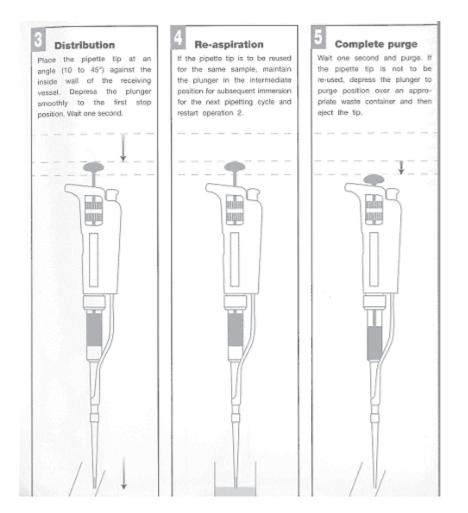


Figure 3 Different steps involved in reverse mode pipetting (cont'd)

Testing pipetting precision

The precision of pipetting should be evaluated periodically (e.g.3 monthly or the lab can establish its own frequency) to ensure the accuracy of results. All records of this evaluation procedure must be retained for quality assurance purposes.

- Using the reverse pipetting technique, pipette 10 replicates of blood and record the weights. Select a volume normally used in the performance of the assay.
- Using the reverse pipetting technique, pipette 10 replicates of bead suspension and record the weights (this applies to methods in which the beads must be pipetted into the tubes).
- Calculate the mean, standard deviation, and coefficient of variation (CV). The CV for replicates should be <2% (Table 1).

Testing pipetting accuracy

The following procedure can be used to test the pipette and how accurately it measures volume. Water is used because the weight of 1 μ L of water is 1 μ g.

- Using the reverse pipetting technique, pipette 10 replicates of distilled water and record the weight.
 (100 µL of water should weigh 0.1000 grams.)
- Calculate the mean, standard deviation, and CV. The CV must be <2% (range: 0.098–0.102).

TO EVALUATE :	ACCURACY	PRECISION	PRECISION	
Replicate #	100 µl of water (grams)	100 µl of blood (grams)	100µl of microbeads (grams)	
1	0.1036	0.1072	0.1056	
2	0.1018	0.1071	0.1056	
3	0.1020	0.1067	0.1055	
4	0.1026	0.1069	0.1056	
5	0.1008	0.1067	0.1052	
6	0.1002	0.1060	0.1055	
7	0.0989	0.1072	0.1056	
8	0.1019	0.1090	0.1047	
9	0.1009	0.1070	0.1050	
10	0.1027	0.1066	0.1050	
Mean	0.1015	0.1070	0.1053	
S.D.	0.0014	0.0008	0.0003	
% CV	1.35	0.73	0.31	

Table 1. EVALUATION OF PRECISION AND ACCURACY

Other Recommendations :

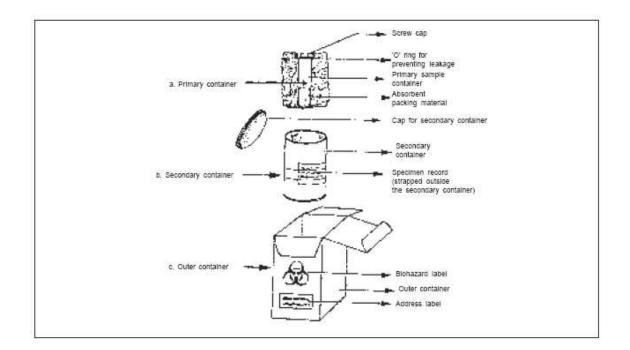
- To ensure optimal performance, the temperature of the pipetted solution and the pipette and tips should be the same (volume errors may occur because of changes in air displace-ment and viscosity of the liquid). Do not pipette liquids with temperatures >70°C.
- Volume errors may also occur with liquids that have a high vapor pressure or a density/viscosity that differs greatly from water. Water is most commonly used to calibrate pipettes and to check inaccuracy and imprecision. A pipette could possibly be recalibrated for liquids with densities that vary greatly from that of water.

- Pipettes should be checked regularly for precision and accuracy.
- Regular maintenance (e.g., cleaning) should be performed either by the user or a service technician according to manufacturer's instructions.
- All calibration and maintenance records must be maintained.

ANNEXURE

APPENDIX 3

An example of specimen packaging for shipping / transport to the CD4 testing laboratory.



APPENDIX 4

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CD+ T- LYMPHOCYTE COUNT REQUEST AND RESULT FORM

1	Patie	ent ID:													Sex:	Μ	
Nam	n e/Oth	er ID:						Year	r of B	irth:					Age:		
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5													-	-		-	
	Clini	c Name):										Tube	add	itive:	EDT	'Α
	-	c Name I 2 : LA		ΑΤΟ	RY U	SE OI	NLY	<u> </u>	~				Tube	add	it iv e :	EDT	Α.
	CTION		BOR		RY U	SE OI	NLY		9	Was	a res	ult pr			itive: this sa		
SEC	CTION	12:LA	BOR		RY U.	SE OI	NLY		9	Was Y	a res N	1		d for	this sa		
SEC	Sam	12:LA	NBOR num l	ber:				/yy):	9	Was Y		1	oduce	d for	this sa		
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APPENDIX 5

FREQUENCY OF CD4⁺ T - LYMPHOCYTE ENUMERATION IN AN HIV-INFECTED PATIENT

Base line CD4 test is propsed for following sub groups of patients

- 1. Stage 3 and stage 4 patients
- 2. Time period of 6 to 8 years after HIV detection
- 3. Patients with history of pulmonary TB and/or herpes zoster
- 4. If tested from outside and CD4 count is less than 350 cells/mm³
- 5. HIV infected partners of those with AIDS.
- 6. All pregnant infected women
- 7. All infants and children below 5 years will be subjected to %CD4 count and absolute CD4 count shall be done in children above 5 years.

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- 6. Dr. Dimple Kasana, former Deputy Director (R&D), NACO
- 7. Dr. Ramesh Paranjape, Officer-in-Charge, NARI, Pune
- 8. Dr. Madhuri Thakar, NARI, Pune
- 9. Dr. V. Ravi, NIMHANS, Bangalore
- 10. Dr. Anita Desai, NIMHANS, Bangalore
- 11. Dr. C. Sokhey, Advisor CDSCO, New Delhi
- 12. Dr. Brajachand Singh, RIMS, Imphal
- 13. Dr. Preeti Mehta, KEM, Mumbai
- 14. Dr. Frank Mandy, QASI, Canada
- 15. Dr. Mario Tamburini, WHO, SEARO
- 16. Dr. Sanjay Sarin, former Lab Specialist, Clinton Foundation
- 18. Dr. Usha K Baveja, former Lab Specialist, Clinton Foundation
- 19. Dr. Trevor Peter, Clinton Foundation
- 20. Dr. Namita Singh, Clinton Foundation
- 21. Dr. S. N. Misra, Clinton Foundation

National AIDS Control Organisation

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