सत्यमेव जयते

National Guidelines for Enumeration of CD4

NACO

National AIDS Control Organisaiton
Ministry of Health & Family Welfare, Government of India

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July 2015

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भारत सरकार
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Ministry of Health & Family Welfare
National AIDS Control Organisation
9th Floor, Chandralok Building, 36 Janpath, New Delhi - 110 001

Message

The enumeration of CD4+ T lymphocytesis of great importance in determining the eligibility for initiation of treatment of ART in asymptomatic people living with HIV. Therefore it is essential that the estimation of CD4 is done accurately and reliably at the designated laboratories.

At present we have around 254 testing laboratories for CD4 estimation catering 450+ ART centres. An effort is also being made that services of this testing of CD4 is provided to the beneficiary near to their places of residences so that they have to travel less for getting their testing done. In this direction a step has already been taken and 20 Point of Care machines have been made operational in remote areas to reduce the inconvenience to the patients for getting this tests done for initiation and monitoring of ART.

This guideline has been made user friendly and provide assistance to the user by many examples and SOPs given in this guideline.

Further in line with the 90-90-90 target for controlling the HIV/AIDS epidemic, that is 90% the proportion of people to know they have HIV, 90% of them on antiretroviral treatment, and 90% of them with suppressed viral load, NACO will continue to expand availability of testing, care and treatment services along with adherence to quality standards. It is hoped that this manual will serve as the guiding document for resolving any inconsistencies that may be existing in testing and ultimately clients will benefit from compliance to this guidance and receive quality assured testing services, resulting in appropriate care and treatment .

I congratulate Lab Services Division for revising the technical aspect of guidelines for Cd4 estimation so that the testing can be made operational in a standardarized qualitative manner all across the centres.

(K.B. Agarwal)

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Preface

Antiretroviral therapy (ART) is estimated to have saved around 1.5 lakhs lives since its introduction in the national programme, a decade ago. Currently, there are over 8.5 lakh people living with HIV (PLHIV), under ART coverage in the country. This includes a sub-set of over 45,000 children with HIV. Enumeration of CD4+ T lymphocytes is critical for ascertaining eligibility for ART initiation as well as assessing treatment effectiveness. About 254 CD4 testing machines are operational at ART centres, servicing the needs of PLHIV for receiving appropriate life-saving care and treatment.

The National AIDS Control Organization (NACO) has undertaken measures to ensure quality of CD4 testing such as a National External Quality Assessment Scheme (NEQAS) for enumeration of CD4+ T lymphocytes with National AIDS Research Institute (NARI) as the apex laboratory and EQA provider. In order to ensure seamless implementation of the NEQAS and continual capacity building of personnel involved in enumeration of Cd4+ T lymphocytes, 13 regional centres are functional across the country to provide customized support to the ART centres.

This manual provides a detailed description of the purpose, pre-requisites and processes for enumeration of CD4+ T lymphocytes. The information provided through this manual will ensure: 1) uniform and correct understanding of enumeration of CD4+ T lymphocytes purpose, pre-requisites and processes by users and 2) consistency in and harmonization of testing procedures in testing centres country-wide. The availability of this manual builds on the ongoing work of NACO on quality assurance in laboratory services.

I thank all contributors and experts for their hard work in producing a very useful document for all CD4 testing laboratories.

(Dr. Naresh Goel)

एड्स का ज्ञान : बचाए जान
TALK AIDS : STOP AIDS

Abbreviations

ADCC	Antibody Dependent Cell Mediated Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
ALC	Absolute Lymphocyte Count
AMC	Annual Maintenance contract
ART	Anti Retroviral Therapy
ATT	Anti Tuberculosis treatment
BD	Becton Dickinson
CD	Cluster of Differenciation
CDC	Centers for Disease Control and Prevention
CMC	Comprehensive Maintenance Contract
COE	Center of Excellence
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
FEFO	First Expiry First Out
FITC	Fluorescein Isothiocyanate
FL	Fluorescence
FSC	Forward Scatter
GLP	Good Laboratory Practices
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSC	Hematopoietic Stem Cells
HTLV-1	Human T-cell Lyphotrophic Virus -1
ICTC	Integrated Counseling and Testing Centre
ISO	International Organization for Standardization
IATA	International Air Transport Association

Abbreviations

LED	Light Emitting Diode
NABL	National Accreditation Board for Testing and Calibration Laboratories
NACO	National AIDS Control Organization
NARI	National AIDS Research Institute
NK	Natural Killer Lymphocytes
NRL	National Reference Laboratory
OI	Opportunistic Infection
PE	Phycoerythyrin
PLG	Panleukogating
PLHA	People living with HIV and AIDS
PMT	Photomultiplier Tube
PEP	Post exposure prophylaxis
POC	Point of Care
PPE	Personal Protective Equipment
QA	Quality Assurance
QASI	Quality Assessment and Standardization for Immunological measures relevant to HIV/AIDS
QC	Quality Control
RNA	Ribonucleic Acid
RBC	Red Blood Cells
SACS	State AIDS Control Society
SOP	Standard Operating Procedure
SPT	Single Platform Technology
SSC	Side Scatter
UKNEQAS	United Kingdom National External Quality Assessment Scheme
UN	United Nations
UPS	Uninterrupted Power Supply
WBC	White Blood Cells
WHO	World Health Organization

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Overview

Since the beginning of the epidemic, almost 60 million people have been infected with Human Immunodeficiency Virus (HIV) and 25 million people have died of Acquired Immunodeficiency Syndrome (AIDS). in 2014 India has the third highest estimated number of individuals infected by HIV/AIDS in the world, after Nigeria and South Africa, with an estimated 2.1 million people living with HIV/AIDS (PLHA).

HIV is a single stranded Ribonucleic Acid (RNA) Lentivirus belonging to the family Retroviridae. Two common genotypes of HIV are HIV-1 and HIV-2, with HIV-1 infection being more prevalent. The natural history of the HIV-1 infection encompasses an acute/ primary phase that lasts for weeks, followed by a clinically latent (asymptomatic) phase that typically lasts for a few years and with the progression of the infection, the immune system is collapsed leading to AIDS.

Lymphocytes are an important part of the cellular blood components and the immune system. All cellular blood components are derived from hematopoietic stem cells (HSCs), which reside in the medulla of the bone marrow (Figure 1.1).

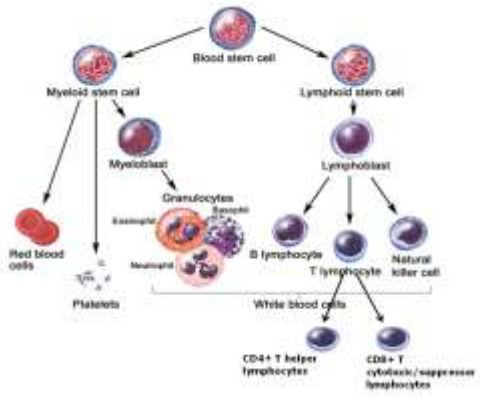

Figure 1.1: Hematopoietic System

HSCs have the unique ability to give rise to different mature blood cell types and are self renewing. Blood cells are divided into three lineages: Erythroid cells (RBC's), Lymphocytes and Myelocytes (granulocytes, megakaryocytes, and macrophages). Lymphocytes are derived from common lymphoid progenitors by lymphopoiesis and finally differentiated into T-lymphocytes, B-lymphocytes and Natural killer (NK) lymphocytes. The T lymphocytes are further classified into helper (CD4+) and suppressor/cytotoxic (CD8+) T lymphocytes. Lymphocytes are phenotypically and functionally heterogeneous and account for 20-40% of the leucocytes (White blood cells) in adults.

T and B-lymphocytes are the major cellular components of the adaptive immune response; i.e., a response specific to the pathogen. T lymphocytes are involved in cell-mediated immunity, whereas B-lymphocytes are primarily responsible for humoral immunity (production of antibodies). In response to pathogens, the T helper cells (CD4+), produce cytokines that direct the immune response while the cytotoxic T cells(CD8+) lyse the pathogen infected cells. Following activation, B cells and T cells leave a lasting legacy of the antigens they have encountered, in the form of memory cells. Throughout the lifetime of an individual, these memory cells "remember" each specific pathogen encountered, and are able to mount a strong

and rapid response if the pathogen is detected again.
Lymphocytes are identified by markers on the surface of their cells called cluster of differentiation (CD). CD is a protein expressed on the surface of the cells of the hemopoietic system. Over 300 CD molecules have been reported so far. These proteins are often associated with specific function of the cells. Cells with different functions express different CD molecules (e.g., CD3+ cells reflect all T lymphocytes, CD4+ cells are T helper cells, CD8+ cells are cytotoxic T lymphocytes, and CD19+ are B lymphocytes).

CD4+ T lymphocytes are the primary targets of HIV. HIV infects these cells by binding to the CD4 molecule expressed on these cells through the gp120 envelope protein resulting in the destruction of CD4+ T lymphocytes. These cells are replenished partially by the host, however, during the course of HIV infection, the replenishment of CD4+ T lymphocytes cannot keep pace with the loss of the lymphocytes. As a result, there is a gradual decrease in the number of CD4+ T lymphocytes in the peripheral circulation. The progressive loss of CD4+ T lymphocytes eventually results in the loss of an ability to develop an adequate immune response to any pathogen leading to opportunistic infections and AIDS.

From the time a person becomes infected with HIV, it may take three to six weeks for anti-HIV antibodies to appear in the peripheral circulation. This period is referred to as the 'window period'. The diagnostic tests that detect anti-HIV antibodies are negative during this period. During this phase there is a surge of viremia, with plasma viral loads reaching a peak in 2-3 weeks and concomitant loss of CD4+ T lymphocytes leading to a transient drop in circulating CD4+ T cells. The host generates an immune response during this period that helps in controlling the viral multiplication leading to a sharp decline in the plasma viral load. The symptoms of the acute primary HIV infection are usually short lived. About 6-12 months post-infection, a steady state of viremia (viral load set point) is achieved. The plasma viral load level remains stable for several years after reaching the set point. The plasma viral load set point is prognostic marker for HIV disease progression. A low plasma viral load set point is usually associated with slower disease progression. Although plasma viral load levels do not rise much during the asymptomatic phase of HIV infection, there is a constant multiplication of the virus leading to the destruction of CD4+ T lymphocytes. The gradual decrease in CD4+ T lymphocytes ultimately results in a loss of control over the immune response and various opportunistic infections start appearing. This is the terminal stage of HIV infection and is called AIDS. Hence, plasma viral load and CD4+ T lymphocyte counts are two important parameters for monitoring HIV disease progression. Currently the estimation of peripheral CD4+ T lymphocyte counts is used for making a decision on the initiation of ART and for monitoring its efficacy in the national program.

The CD4+ T lymphocytes are commonly estimated using the combination of monoclonal antibody and flowcytometry. The technologies based on both flow cytometry and non-flowcytometry principles are available and will be discussed in this guideline.

Role of CD4+ T Lymphocytes in Disease Progression

The primary receptor of HIV is CD4 antigen. HIV can infect any cell bearing the CD4 antigen on its surface. This primarily pertains to CD4+ (helper/inducer) T lymphocytes and other cells expressing a few molecules of the CD4 antigen on their surface(e.g., cells of monocyte–macrophage lineage, microglia, astrocytes, oligodendrocytes, placental tissues containing villous Hofbaurer cells, cells of the colon and cervix). HIV infects CD4+ T lymphocytes resulting in the progressive destruction of these cells.

Mechanism of CD4+ T lymphocytes dysfunction and depletion

The changes in CD4+T lymphocytes in HIV infection are both quantitative and qualitative and affect virtually every limb of the immune system. The reduction in the number of CD4+ T lymphocytes is directly due to the infection by HIV and also indirectly due to the killing of uninfected cells.

Direct mechanism of CD4+ T cell depletion

- Loss of plasma membrane integrity due to viral budding.
- Syncytia formation.

Indirect Mechanism of CD4+ T Cell Depletion

- CD4-gp120-mediated fusion of infected and uninfected cells.
- Super antigen mediated activation of T cells, rendering them more susceptible to infection from HIV. Innocent bystander killing of viral antigen coated cells.
- Apoptosis due to functional exhaustion.
- Elimination of HIV uninfected cells by ADCC (Antibody Dependent Cell Mediated Cytotoxicity)when the HIV antibodies recognize the CD4-gp120 complex.

Absolute CD4+ T cell Counts and Percentages

Absolute CD4+ T Cell Count: Absolute CD4+ T cell count is the enumeration of the number of CD4+ T cells circulating in the blood. This is reported as the number of CD4 cells/µL or cubic mm (mm3) of blood. The absolute CD4+ T cell count enumeration in an HIV infected individual is used to monitor the disease progression. It is also used to decide on the initiation of ART as well as for monitoring its efficacy. Absolute CD4 counts in healthy adults are usually between 600-1800 cells/cu mm.

Factors that affect absolute CD4 count:

The absolute CD4 count is calculated based on the total white blood cell (WBC) count and the percentages of CD4+ T lymphocytes. Hence this absolute number may be influenced by factors that may affect the total WBC count and lymphocyte percentages, such as the presence of acute infections, splenectomy or co-infection with human T-lymphotropic virus type I (HTLV-1).

Diurnal variation in CD4 counts with lower CD4 counts in the morning has been observed in human beings irrespective of the HIV status. The absolute CD4 counts have shown week- to-week and seasonal variations. The changes in CD4 count may also be seen related to exercise, smoking, inter-current infections and some medications other than ART (eg corticosteroids). The intra-subject variability due to the physiological conditions might account for variations in the CD4 counts as much as, assay related variations.

CD4+ T Cell Percentage: A CD4 + T cell percentage represents the proportion of CD4+ T cells of total lymphocytes. The absolute CD4+ T cell count might be influenced by an increase or decrease in the absolute lymphocyte counts. Hence, CD4 percentages could be used as a reliable indicator for the effective function of the immune system. It is recommended by some scientists to include the CD4 percentages in the guidelines for ART. However, at present the CD4 percentage is used only for the assessment of immune dysfunction in the pediatric population.

The HIV infected individuals are monitored for the disease progression and to decide the initiation of ART using the CD4 + T cell counts. The clinical staging of an HIV infection is also used to decide on the initiation of ART. According to the National AIDS Control Organization (NACO) guidelines, ART should be initiated if the CD4 + T cell count drops below 350 cells/mm3. Table 2.1 outlines the criteria for initiation of ART in Adults and Adolescents using CD4 counts and WHO clinical staging of infected individuals.

Table 2.1: Initiation of ART based on CD4 count and WHO clinical staging

WHO Clinical Staging	CD4 (cells/mm3)
HIV Infected Adults & Adolescents	
Clinical stage I and II	Start ART if CD4+ T cell count <350 cells/ mm3
Clinical stage III and IV	Start ART irrespective of CD4+ T cell count
Pregnant Women	
All positive pregnant women	Start ART irrespective of CD4+ T cell count
For HIV and TB co-infected Patients	
Patients with HIV and TB co-infection (pulmonary/ extra-pulmonary)	Start ART irrespective of CD4+ T cell count or the type of tuberculosis (Start Anti Tuberculosis Treatment (ATT) first, initiate ART as early as possible between 2 weeks to 2 months, when TB treatment is tolerated)
For HIV and Hepatitis B and C (HBV/HCV) co-infected Patients	
HIV and HBV/HCV co-infection– without any evidence of chronic active Hepatitis	Start ART if CD4 < 350 cells/ mm3
HIV and HBV/HCV co-infection– with documented evidence of chronic active Hepatitis	Start ART irrespective of CD4+ T cell count

Table 2.2: CD4 monitoring and follow-up schedule

Pre ART CD4 monitoring and Follow up Schedule	
CD4 Count	**Timing**
Between 350 and 500 & not on ART	Every 6 months
>500 & not on ART	6 months
CD4 of any value and on ART	Every 6 months
Note: If the CD4 count is between 350 to 400 cells/mm3 and the patient is not on ART; repeat CD4 assessment after 4 weeks.	

Opportunistic infections and CD4+ T cell counts:

An opportunistic infection (OI) is a disease caused by a microbial agent in the presence of a compromised host immune system. The appearance of many OIs correlates with the drop in the CD4+ T cell count as depicted in the figure 2.1.

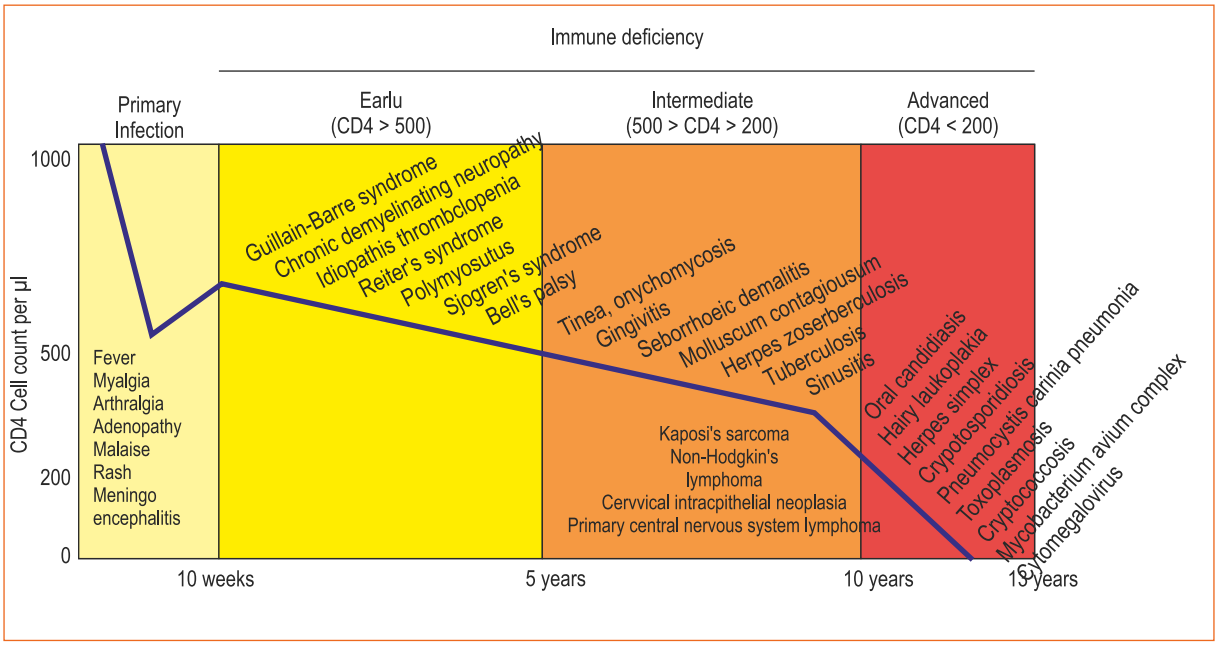

Figure 2.1: Association between OIs and CD4+ T lymphocyte count

Note: *Tuberculosis can be associated with any CD4+ T cell count*

CD4+ T Cell Enumeration in the Pediatric Population

HIV infection in children progresses more rapidly than in adults, and some untreated children die within the first two years of life. This rapid progression is correlated with a higher viral burden and faster depletion of CD4+ T lymphocytes. The absolute CD4+ cell count is variable in children and they normally have a relative lymphocytosis. The counts in healthy children are much higher than adults and slowly decline to adult levels by 5 years of age. CD4+ T cell

percentage varies less with age and is a better immunologic parameter for following children up to 5 years of age. Table 2.3 gives the classification of HIV-associated immune dysfunction and age related CD4 counts in HIV infected children.

Table 2.3: WHO immunological classification for established HIV infection

Classification of HIV-associated immunodeficiency	Age related CD4 values			
	CD4%			CD4 count
	Age <11 months	Age 12-35 months	Age 36-59 months	> 5 years
Normal	>35	>30	>25	>500
Mild	30-35	25-30	20-25	350-499
Advanced	25-30	20-25	15-20	200-349
Severe	< 25% or CD4 count <1500 cells/cubic mm^3	< 20% or CD4 count <750 cells/cubic mm^3	< 15% or CD4 count <350 cells/cubic mm^3	< 15% or CD4 count <200 cells/cubic mm^3

Ideally CD4 percentages directly obtained from the CD4+ T cell enumerating equipment should be used, however, in case equipment gives only absolute CD4 counts, the CD4 percentage should be calculated using the formula below.

CD4 Percentage= absolute CD4 count/Total lymphocyte count* x 100

*Total lymphocyte count is obtained from the hematology analyzer; however, it may add variation to the CD4 count

Absolute CD4+ T cell count is used for monitoring the disease progression in children above 5 years of age.

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Principles of flow cytometry

3.1 Introduction:

Flow cytometry immuno-phenotyping follows several analytical steps: Cells are first identified by light –scattering properties ,then further defined by the binding of fluorochrome –tagged monoclonal antibodies By combining the light scatter and fluorescence information about each and every cell, populations of cells with common characteristics can be identified and quantitated.

3.2 Flow cytometry

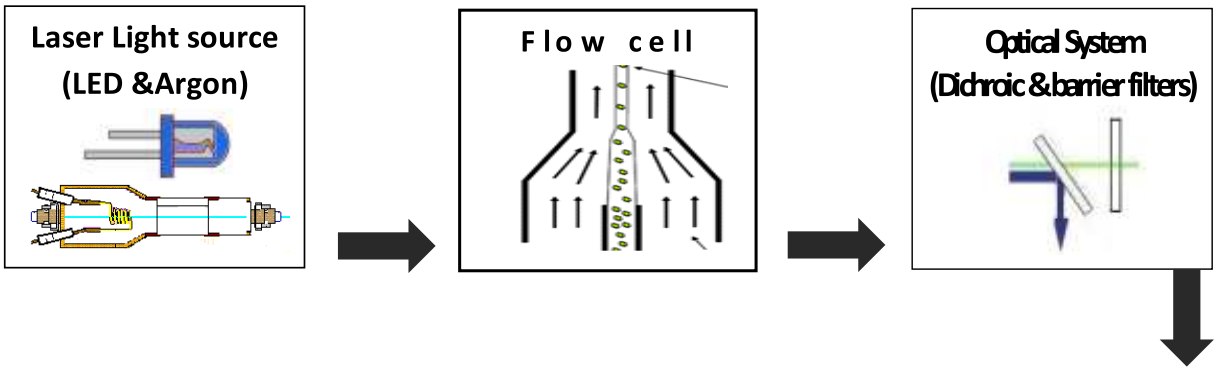
Flow cytometry refers to a technology that simultaneously measures and analyzes multiple physical and chemical characteristics of single cells or other biological particles, as they flow in a fluid stream past optical and/or electronic sensors. It provides information about their relative size, relative granularity or internal structure, and fluorescence in several spectral regions emitted by fluorochrome labeled probes which bind specifically and stoichiometrically to cellular constituents such as protein antigen and nucleic acids.

The major applications of this technology are:

1. Identification of cells
2. Cell Sorting

Flow cytometry is the measurement and analysis of cells (cytometry) in a fluid system (flow), which delivers the cells singly past a point of measurement to which light, is focused. The light scattered by the individual cells is recorded by the optical sensor which converts the light signals into electronic signals measured by the electronic and computer system of the flow cytometer (Fig 3.1). Besides, the fluorescence emitted by the cells stained with fluorochrome labeled antibodies specific to the cell receptors expressed on the surface can be measured and used to sort the cells. Cells can be sorted from the main cellular population in to subpopulation for furthur analysis. Fluorescence activated cell sorters(FACS) sort cells according to specified parameters such as intensity of fluorescence and size.

The unique advantage of Flow cytometry is that they can rapidly and quantitatively measure multiple simultaneous parameters on individual live cells.



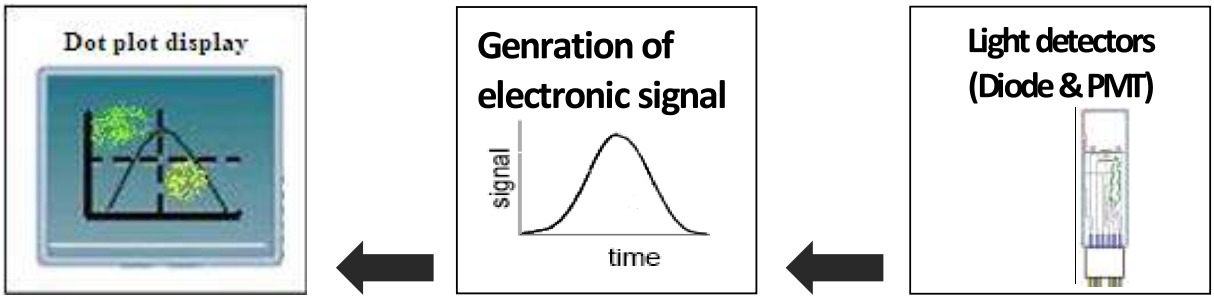


Figure 3.1: Staining process in flow cytometry

The degree and the direction of scattering of light by single cells depends on the size (measured by forward scatter: FSC), granularity and the internal structure (measured by side scatter: SSC) of the cells. The phenotypes of the cells being analyzed are identified using the fluorescent tagged antibodies against the specific receptors on the cell surface such as lymphocytes, neutrophils, eosinophils, basophils and monocytes. (Fig 3.2)

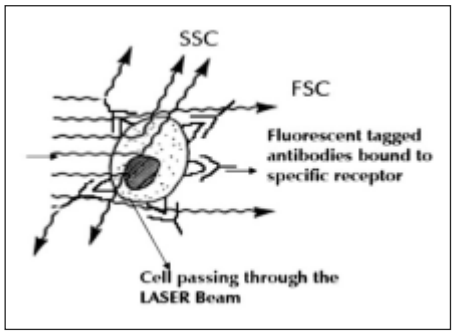


Figure 3.2: Light Scattering and fluorescence in flow cytometry

3.3 Components of flow cytometry

The system consists of three basic elements. 1. Fluidics (Flow chamber) 2. Optics and 3. Electronics.

Fluidics: Flow chamber (cell): The flow chamber lies at the heart of the instrument. It is designed to deliver the cells in a single column at the point of measurement (through laser beam).For optical illumination, the stream transporting the particles should be positioned in the centre of the laser beam. The portion of the fluid stream where cells are located is called the sample core and the surrounding fluid is called sheath or sheath fluid. As the sample enters the flow cell chamber, the outer, faster flowing sheath fluid hydro-dynamically focuses sample fluid into a narrow core region within the jet and presents a single cell/particle to excitation sources (fig: 3.3). Any cell ranging from 0.2 to 100 µm in size is suitable for flow cytometer analysis. The cells are ejected through this flow chamber at a rate of approximately 1000 cells/sec.

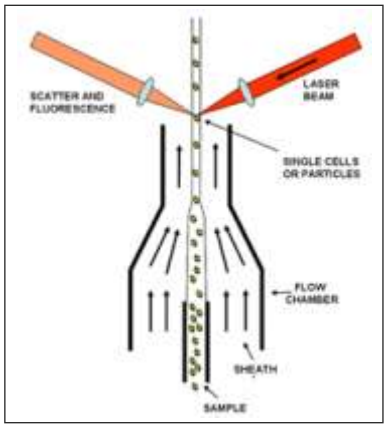


Figure 3.3: Hydrodynamic focusing of the sample core through a flow cell

The rate of the sample flow is regulated by air pressure regulator that may be fixed to three settings (high, medium and low). A higher flow rate has less resolution and is generally used for procedures like immune phenotyping (for example, CD4+ or CD8+ T cells). A lower rate is generally used in applications where greater resolution is required, such as DNA analysis.

Optics:
The optics system consists of excitation or illumination optics and collection optics. The excitation optics consists of light sources and lens.

Light sources: Light sources can be either laser (e.g. an argon ion laser with 488 nm single line emission), or conventional lamps (e.g. mercury arc lamp) or Light Emitting Diode (LED). Lasers produce a single wavelength of light (a laser line) at one or more discrete frequencies (coherent light). Laser is used in most of the flow cytometers for the following reasons:

1. Light output from laser is of high intensity, monochromatic, unidirectional and appears in phases.
2. The sharp excitation of a single cell minimizes the probability of more than one cell being analyzed at the same time.

There is a large variety of air-cooled and solid state lasers available. The most common primary laser is an air-cooled argon-ion laser producing blue light at 488 nm. This wavelength is convenient for the excitation of fluorescein, the first immune fluorescent dye to be used.

Lenses: The focusing of laser beam onto the sample stream is accomplished by a simple lens giving a beam cross-section of, typically, about 50 µm.

All of the signals are routed to their detectors via a system of mirrors and optical filters.

Dichroic mirror: Dichroic mirrors are a type of beam splitters that direct light of different wavelengths in different directions. .A dichroic filter is a very accurate color filter used to selectively pass light of a small range of colors with long wave length while reflecting colors of short wave length (figure 3.5). Simultaneous use of many dichroic filters can enable the flow cytometer to measure the fluorescent signals emitted by different cells and subsets of cells in a single setting.

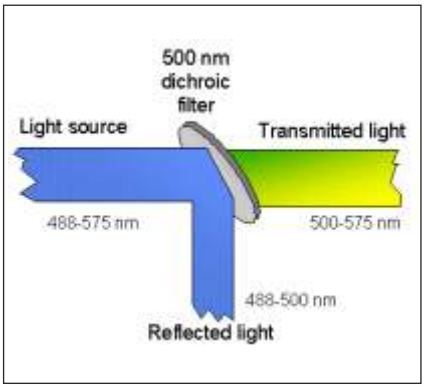


Figure 3.5: Dichroic mirror

Barrier Filter: The role of the barrier filter is to block any reflected excitation light of short wave engths and transmit only the fluorescence from the cell.

Photo detectors: The collection optics or photo detectors consists of a collection lens to collect light signals emitted from the cells In general, flow cytometer has three types of photo detectors:

Forward Scatter detector (FSC) is a relatively sensitive silicon photodiode set in the main part of the beam. The intensity of this scatter signal depends on the cross-sectional area of the cell (i.e. the size) and not upon its refractive index.

Side scatter detector (SSC) is a photomultiplier tube that receives refracted and reflected light signals which are proportional to cell granularity or complexity. This SSC is detected at 90º to incident light axis.

Fluorescence detector (FL): It is also a photomultiplier tube that receives fluorescence emitted by the cells

Photomultiplier tubes (PMT): PMTs are extremely sensitive detectors of light in the visible, ultraviolet, and near-infrared ranges of the electromagnetic spectrum. The current produced due to the light signals and fluorescence is multiplied 100 million times enabling individual photons to be detected when the incident flux of light is very low. In a flow cytometer layout (figure 3.6), separate fluorescence (FL) channels are used to detect the light emitted by the particles/cells passing the laser beam.

- The first dichroic mirror reflects blue light (below 500 nm). After passing through a barrier filter which selects blue light (480-500 nm), the light falls on the side scatter detector (SSC). The second dichroic mirror reflects green light (below 560 nm). After passing through a barrier filter which selects green light (510-550 nm), the light falls on the FL1 PMT.
- The third dichroic reflects yellow light (below 580 nm) which passes through a yellow barrier filter onto FL2 PMT.
- The fourth mirror reflects orange light (below 600 nm) which passes through an orange filter (550-600 nm) to FL3 PMT.
- The long wave red light passes through the dichroic mirrors without reflection and falls on the FL4PMT.

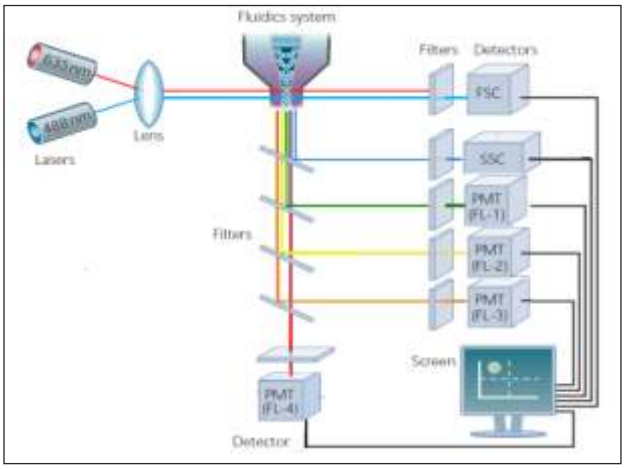


Figure 3.6: Layout of optical filter

Electronics: The electrical pulses originating from light detected by the Photomultiplier tubes (PMTs) are then processed by a data processor using a series of linear and logarithmic amplification.

Signal processing

Pre amplification: When light hits a photo detector, a small current (a few microamperes) is generated. Its associated voltage has amplitude proportional to the total number of light

photons received by the detector. This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically (figure 3.7).

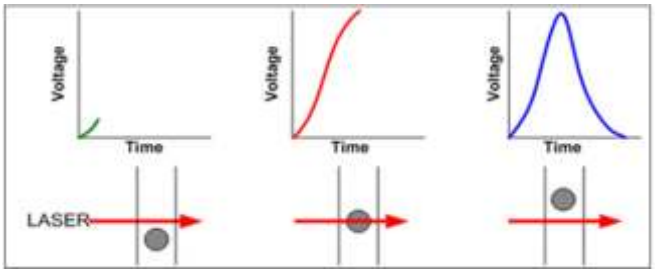


Figure 3.7: Generation of signal from the flow cell

Linear amplification is often used to amplify FSC and SSC light signals of cells and the logarithmic amplification is most often used to measure fluorescence of cells. Log amplification expands weak signals, separates negative signals from dim positive signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram.

Display of the data by digital Processing: It is the trigger setting of a flow cytometer so that 'signal' derived light scatter from the particle of interest (for example, a cell) or a fluorescence emitted from the cell is received and the 'noise' in the form of debris and 'spikes' from electronic noise is ignored. A threshold level is set on one, or possibly two, parameters such that a cell is only detected when the signal rises above this level. The schematic is shown in the figure 3.1.

Data Analysis: All flow cytometers have inbuilt or external computer . The computer program helps in data acquisition and analysis by performing the following functions

- select the parameters for measurement;
- select area, width or height on different parameters
- adjust the voltages on the PMTs;
- adjust the gain settings on the amplifiers;
- select logarithmic or linear amplification
- select and adjust the threshold (discriminator) settings
- adjust the settings for colour compensation
- select histograms and cytograms for display;
- draw regions and set gates.

As data are acquired data file is created on the hard drive, often referred to as 'listed data'.

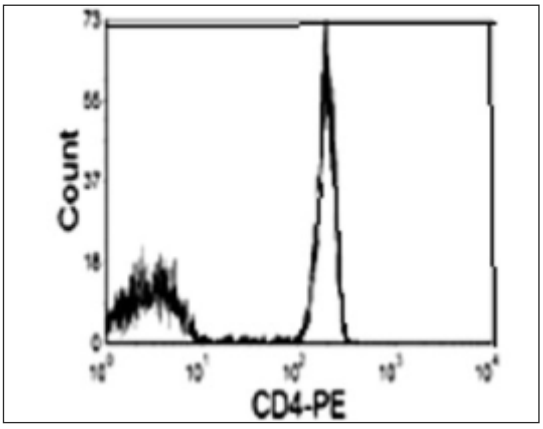


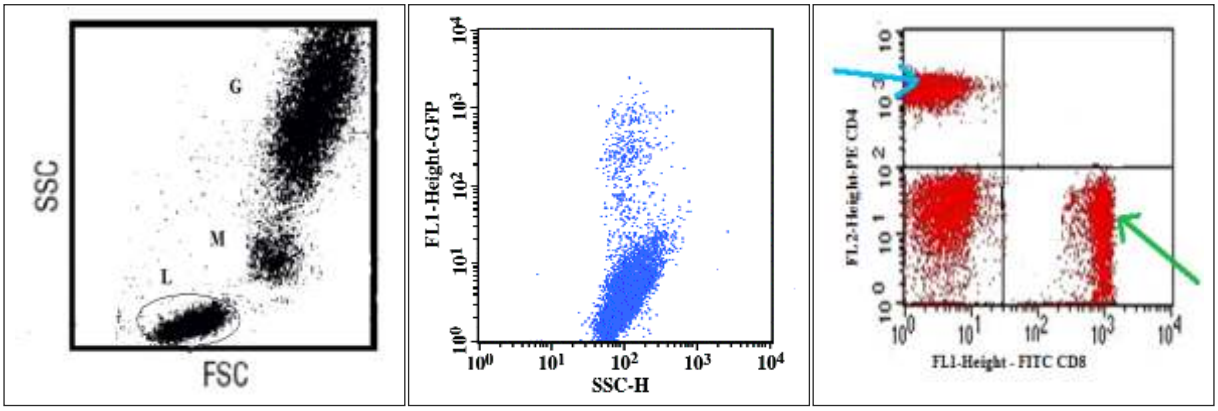
Figure 3.9: Histogram: single parameter

Data display and visualization:

Flow cytometry data can be displayed as histogram, 3-dimension, and contour plots as well as density plot.

A histogram (Figure 3.9) shows varying values for one measured parameter, the fluorescence signal emitted by the PE dye- monoclonal antibody bound to the CD4+ cells shows only the collection of events (number of cells). It does not show the variances in expression of the event.

A scatter plot, dot plot shows the relative values of two different parameters as they relate to each other. The commonly used dot plot or the scatter plots are shown in the Figures 3.10 A to C.



A:Scatter plot: FSC vs SSC | B:Single color fluorescence vs.SSC | C:Two color fluorescence

Figure 3.10 Scatter/ Dot plot

1. **Forward scatter vs. side scatter** (Fig 3.10 A) defines three distinct cell populations on the basis of size and granularity; these are the granulocytes, monocytes and lymphocytes.
2. **Single color fluorescence vs. SSC** (Fig:3.10 B) shows not only the absolute number but also the variances in the expression of the fluorescence of the cells.
3. **Two color fluorescent dot plot** (Fig: 3.10 C) shows a sample that has some single positives for FITC (CD8+cells)along the x-axis (green arrow) and some single positives for PE(CD4+cells) along the y-axis (blue arrow).This plot helps to gate around & narrow the analysis of the cells expressing either or both markers.This plot also helps to discriminate dead cells from the live ones that are expressing the desired fluorescence

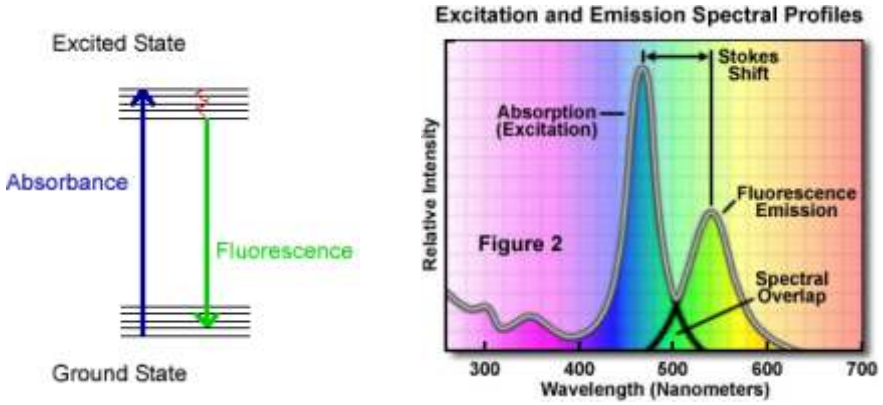
3.4 Use of fluorochrome in flow cytometry:

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength. In most cases, emitted light has a longer wavelength, and therefore lower energy than the absorbed radiation. The excitation-emission pattern of fluorescence at molecular level (figure: 3.8 a) shows the following steps:

1. Absorption of light energy by a molecule (Blue arrow).
2. The molecule "accommodates" this additional energy by promoting an electron to a higher (excited) energy level.(black horizontal lines).
3. Loss of energy through enhanced vibrations of the molecule i.e. Stokes Shift phenomenon (red wavy line).

4. Fluorescence occurs when the molecule releases that remainder of the energy by emitting light (green arrow). Because of the energy loss due to molecular vibration in between the absorbance and emission processes, the fluorescence usually has lower energy (higher wavelength) than the absorbance .

The fluorochromes thus can be coupled with the monoclonal antibodies against to different receptors on the cell (for e.g. CD4) which in turn can attach to the cells recognizing the receptors. This property is used to identify the cells using the specific light (color) emitted by that fluorochrome dye. The amount of fluorescent light detected is proportional to the number of fluorochrome molecules on the cells. The two most commonly used fluorochrome dyes are fluorescein isothiocyanate (FITC) and phycoerythrin (PE), among a group of phycobiliproteins. These are easily attached to antibodies by reaction of its isothiocyanate derivatives with protein amino groups. FITC and PE on excitation emit green and orange fluorescence respectively. Hence, both dyes can be used in combination to study two different parameters for e.g. CD4+ (PE) and CD8+cells (FITC)/ T-Lymphocytes (CD3+cells) simultaneously.



(a) (b)

Figure 3.11: Regionalizing scatter plot

Several fluorochromes show spectral overlap of fluorescent emissions (Fig 3.8 b). This leads to incomplete optical separation by filters. This fluorescent overlapping often happens when the same wavelength of excitation is used. This results in mixed or unseparated cell populations. To compensate this overlap, output signal of one fluorochrome is subtracted from the output of the other fluorochrome and vice versa. This electronic subtraction is called 'compensation'. Compensation is very important to get clean display of the cell population of interest.

An example is the popular combination of FITC and PE excited by 488 nm Argon ion laser light. FITC has its peak emission at 525 nm and has around 20%-30% emission contamination in the emission area of PE (peak emission at 575 nm). The PE has about 1%-2% of emission contamination in the emission area of FITC. To compensate this overlapping, a fraction of FITC sensor output signal is subtracted from the output of the PE-output signal and vice versa.

a. Sorting:

Cell Sorting is accomplished using stored computer information. To sort particles or cells, the cytometer needs to identify and separate out the individual cells of interest . Once the population of interest has been identified on a data acquisition plot, a region is drawn around that population. A logical gate is created from the regions. This gate is then loaded in to the cytometer's software as the sort gate .The sort gate identifies cells of interest to be sorted out of the stream.

3.6 Gating strategies:

To visualize the correlations in multi parameter data or to analyze the cell population of interest, 'regions' and 'gates' (R1, R2 etc) are drawn around a population of interest on a one- or two-parameter plot. (Fig.3.12).The gate or region can be drawn while acquiring the data or while analyzing the data after acquisition.

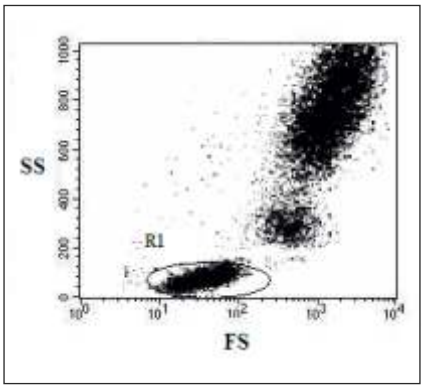


Figure 3.11: Regionalizing scatter plot

Figure 3.11 shows a region (R1) drawn around the lymphocyte cluster in the light scatter cytogram. The advantage of the gate is that once the cell population is gated, it can be identified in all other dot plots created from this data. It is also possible to draw multiple gates to identify and quantify the cell population of interest. The percentage of these cell populations also can be obtained from the software.

Different gating strategies are used in process of identification of population of interest.

FSC Vs SSC gating:

An absolute CD4 T lymphocytes count is then derived using a mathematical formula: (%Cd4+ T-cells x the absolute lymphocyte count). Because the percentage of CD4 T lymphocytes is obtained from the reference lymphocyte populations, the purity of the lymphocyte gate is most essential. Hence, for the sample with a high proportion of lymphocytes, the percentage of CD4 T lymphocytes can be easily derived from a 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 the sample has 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 gates, then this morphological gating remains questionable. Hence, this gating strategy is now considered as unacceptable.

T cell gating

The T lymphocyte gating strategy uses the CD3+ T cell gating using the CD3-specific monoclonal antibodies and the no. of CD4+ CD3+ T cells can be estimated once all CD3+ T cells are gated. A no. of equipments (e.g. FACSCount) use this strategy.

CD45 gating

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. This method uses 2 markers; CD45 and CD14 (CD45 is a pan-leukocyte marker expressed at different intensities on leukocytes (granulocytes Cd45+; Cytotoxic CD45++; lymphocytes CD45+++ or bright) while the CD14 marker is selectively expressed by monocytes). This CD45+++/CD14-backgating can gate all lymphocytes in the acquired events and maximize their purity by excluding unwanted non-lymphocytes.

Advantages of CD45 gating

- Easy differentiation of lymphocytes even in the presence of a large amount of debris, so that the lysed sample can be acquired without an intermittent washing step (lyse/no-wash staining).
- No need to use the isotype control thus saving the cost of the reagents.

However, the use of CD45/CD14 gating strategy is not being used clinically. It has been replaced by a modified gating strategy, known as Panleucogating (PLG) that uses CD45 and SSC for gating

Panleucogating

This 2-colour PanLeucogating uses total leucocytes as the common denominator, in which total leucocytes are identified and gated by their SSC and CD45+ characteristics. After staining with CD45 FITC and CD4¬PE, 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. Lymphocytes gated in this region are further analyzed for CD4 T lymphocytes by using SSC against CD4 T lymphocytes with other fluorochrome. CD4 T lymphocytes are easily distinguished from non-CD4+ T-cells and %CD4 is then obtained as a percentage of total lymphocytes. 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 CD4 testing that is suitable in the resource-poor areas of the world. This approach can be used to test samples up to five days after collection.

Several conventional flow cytometers are available that perform three and four colour analysis for estimation of CD4 T lymphocytes counts using Panleucogating strategies. (Fig. 3.12)

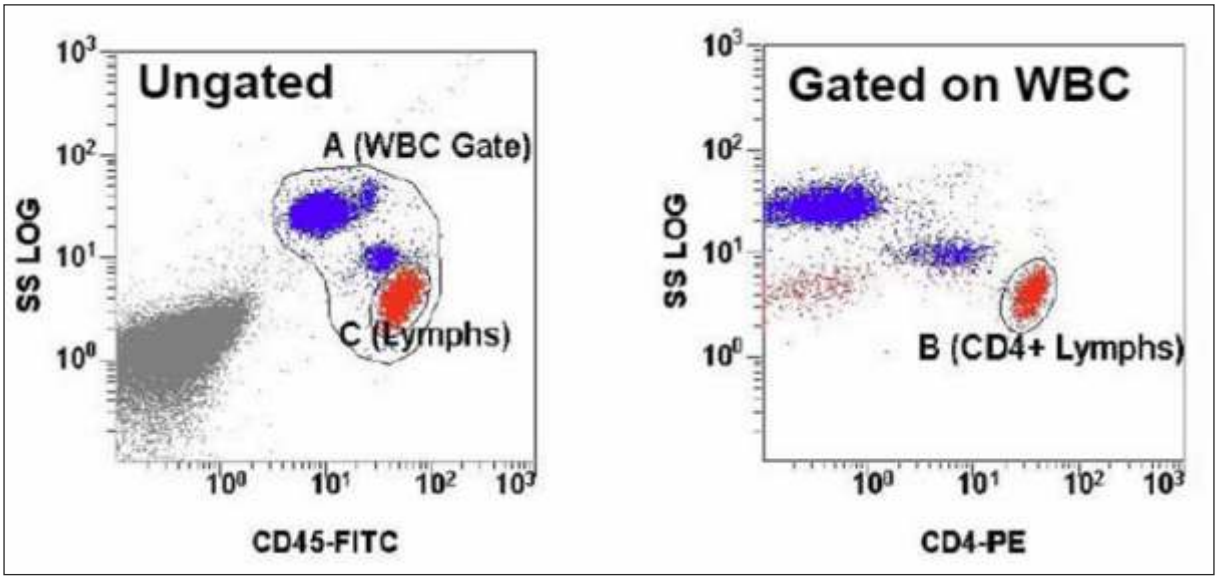


Figure 3.12 Flow cytometric display of the panleucogating approach for CD4 count estimation

The use of the conventional flow cytometer requires extensive training of personnel; good routine maintenance and very good service back up. In laboratories with a large sample load, the use of flow cytometry is desirable.

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Enumeration of CD4+ T cells

Several methodologies for enumerating CD4+ T lymphocyte counts are being used and are under develpmental stage . These are either flow cytometric or non-flow cytometric. The mention of products mentioned in the chapter does not imply that they are endorsed or recommended in preference to others of a similar nature.

Techniques for Enumeration of CD4+ T Lymphocytes

Flow cytometry methods based on the principle of immunofluoroscence are currently the standard technology for CD4+ T lymphocyte counting. They are used because they are accurate, precise, fast and reproducible. The use of the conventional flow cytometer requires extensive training of personnel, good routine maintenance, and very good service back up. In laboratories with a large specimen load, the use of standard flow cytometry is desirable.

Dual Platform Technology

The flow cytometer provides the relative percentages of cells expressing the specific receptor (e.g., CD4). For calculating the absolute counts, it is necessary to obtain the absolute lymphocyte count from the hematology analyzer. This method is known as dual platform technology (DPT). The DPT requires a flow cytometer to generate the percentage of CD4-positive lymphocytes , and a hematology analyzer to obtain the total white blood and lymphocyte counts. The DPT introduces variables from two pieces of equipment and, hence, is not a preferred technology.

Single Platform Technology

The single-platform technology (SPT) enables absolute CD4+ T lymphocyte counts to be derived directly from the CD4 enumerating equipment without the need for a hematology analyzer. This can be assessed either by counting CD4+ T lymphocyte populations in a precisely determined blood volume or by using the known numbers of fluorescent micro beads admixed to a known volume of blood and stained with CD4 monoclonal antibodies. It is required to pipette small amounts of reagents, i.e., 10 µl to 25µl. Hence, the pipetting technique is very crucial for the reliable use of the single-platform technology.

To date, many single-platform technologies are commercially available.

Standard Flow Cytometers as SPT

Standard flow cytometry can be modified into a single platform technology by using the CD45 (marker for all leucocytes) gating approach in combination with fluorescence beads. A known number of fluorescence beads are included in the tubes as a lyophilized pellet that dissolves in a blood sample during sample preparation. By gating the bead population during analysis, absolute cell counts can be readily determined by a simple calculation. The advantage of using a

standard flow cytometer as a SPT is that both the percentages as well as absolute counts can be obtained simultaneously. The determination of both of these clinical parameters is important because the CD4+ lymphocyte percentage is often required for monitoring pediatric HIV-positive populations. The available technologies are TruCOUNT tubes (BD Biosciences), Flow-Count fluorospheres (Beckman Coulter) and Cyflow (Partec) (Figure 4.1).

Figure 4.1: Standard flow cytomters: FACS Calibur, EPICS XL-MCL and Cyflow

Modified Flow Cytometers

- **Microbead-based Systems**

FACSCount (Becton Dickinson Biosciences) (Figure 4.2) is the only available micro bead-based single-platform instrument that is designed specifically for enumerating the absolute CD4+, CD8+ and CD3+ T-cell counts. The whole blood is added to reagent tubes containing antibodies and reference beads; single tube is used for enumeration of the absolute number of CD3+CD4+ T cells and two tubes are used for enumeration of both CD3+CD4+ and &/or the CD3+CD8+ T-cells. After incubation the tubes are placed in the equipment and the stained cell suspension is acquired in the equipment. The fluorochrome labeled reference beads in the reagent tube functions as a fluorescence standard for locating the lymphocytes and also as a quantitation standard for enumerating the cells. The calculation of absolute CD3+, CD4+ and CD8+ T-cells is determined automatically by using the built-in software program.

Figure 4.2: FACSCount microbead-based system

Volumetric systems

- **Millipore Guava EasyCD4 :**

The GuavaEasyCD4 platform (Guava Technologies) is a modified flow cytometry system based on a micro capillary technology (Figure 4.3). The flow cytometer is coupled with a laptop computer for analysis. The system uses a diode green laser as a light source. The system uses the volumetric principle; the T cells are gated to identify CD3+ CD4+ T cells using two monoclonal antibodies, CD3-PECy5 and CD4-PE. The procedure does not require sheath fluid and the system's sampling precision depends on the integrity of the fluid pathway.

Figure 4.3: Guava Easy CD4 Volumetric system

- **Cyflow counter: (Partec)**

Cyflow is another desktop single-platform technology made by Partec, Germany (Figure 4.4). It uses 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 (capable of 2 or 3-color analysis). Data acquisition and analysis are performed in real time with FlowMax software. It can be used as a mobile system that can run on car batteries. The procedure does not require lysing of RBCs or washing.

Figure 4.4: Cyflow Count (Partec)

- **CD4 Testing at the Point of Care**

Cd4 testing needs to be brought closer to the point of patient care to improve access to CD4 testing in resource-limited settings and to bring down the overall program costs. Point of Care (POC) CD4 technologies are designed to produce these results in a very short time at the POC. These devices are portable, battery operated, and easy to use. Availability to this equipment, over time, would make CD4+ T cell counts available at smaller centers and in remote areas. This would bring the testing closer to the patients. These technologies are expected to reduce the number of visits missed by patients, improve adherence to care, and reduce loss to follow up significantly. The POC reagents should have long shelf lives and be stable in conditions of high heat and humidity. This would mean that the frequent deliveries of supplies could be reduced, eliminating the need for a cold chain.

To date, CD4 assay development approaches include selective cell staining, followed by capture or count by digital photography, measuring CD4 molecules instead of cells, or measuring proxy molecules of CD4. There are several CD4 POC technologies in the development pipeline.

Three of these technologies are already on the market; PointCareNOW™, the Pima™ CD4 Analyzer, and the CyFlow™ CD4 mini POC. The remaining technologies discussed, including those from Daktari, Omega, Zyomyx, MBio and others, are not yet in the market.

It is crucial that any new technologies be put through rigorous performance evaluation and validated against the existing reference technologies before being adopted for use in the national program. It is also extremely important that these devices be able to perform well in the external quality assessment for CD4+ T cell enumeration.

The PIMA™ Analyzer

The Pima™ analyzer (Figure 4.5) is a small bench-top, fixed volume cytometer manufactured by Alere™ Inc. The Pima system is made up of the analyzer and a disposable Pima CD4 test cartridge containing dried reagents. It is capable of measuring absolute CD4 counts in whole blood, but it cannot currently determine percentage CD4 counts for pediatric use. The cartridge is able to take up approximately 25 µl of blood, which it then combines in the cartridge with the dried reagents needed to run the test. The Pima CD4 test is actually performed within the cartridge and no part of the Pima analyzer comes into contact with the blood sample during processing. This minimizes the risk of analyzer contamination.

Figure 4.5: Point of Care CD4 enumerating equipments

After loading the sample on the cartridge, the sample interacts with CD3 and CD4 specific monoclonal antibodies, each labeled with a different fluorescent dye in the detection area. CD4+ T cells are identified as CD3+CD4+ T cells. The collected signals within the detection channel are then counted and correlated against the volume of the detection channel. The CD4+ T cell count is displayed within 20 minutes. The Pima analyzer is equipped with miniaturized, multi-color fluorescence imaging optics. Fluorescence signals are detected by an on-board camera and analyzed using proprietary software algorithms on board an embedded computer. T-helper cells carry both CD3 and CD4 surface antigens and therefore emit light at wavelengths specific for both antibody-dye conjugates. This allows for the specific differentiation of T-helper cells from other blood cell types carrying only one of the two surface antigens. The Pima™ analyzer has provisions for data entry (Operator ID, sample ID), archiving, connectivity to printers, and data storage media. All results are saved in an on board archive and may be retrieved and downloaded by the operator at any time after the test is performed. A test report may also be printed using an attached external printer. The Pima system is considered robust and requires minimal operator training. It can perform upto 20 tests per day. As a simplified POC system, it can be used appropriately at all levels of the healthcare system where high-volume throughput is not required or in situations where same day results are particularly important, even in high-volume settings. Maintenance of the instrument is simplified because failed instruments can be swapped out for new systems.

The CD4 PIMA analyzer is WHO prequalified and CE-IVD marked. The company is in the process of obtaining the FDA approval for this product. The literature on the validation of PIMA in diverse settings and geographies has shown that the performance is on par with standard flow cytometers.

PointCareNOW™

The PointCareNOW™ system, (Figure 4.5) developed by PointCare Technologies Inc., is a compact tabletop system that measures CD4 absolute count and CD4 percentages, WBC count and hemoglobin, as well as total leucocytes count and percentage lymphocytes, monocytes, neutrophils and eosinophils. The system uses forward light scattering (rather than the fluorescent dyes used in some systems) to distinguish lymphocytes from white blood cells. It then uses a colloidal gold label to change the natural light scatter characteristics of the CD4 lymphocytes to perform the CD4 enumeration.

The PointCareNOW is a modular fully automated pre-calibrated platform. There are no manual sample preparation steps for pipetting, incubation, vortexing etc. The operator is able to take a capped phlebotomy blood-sample tube and, with the cap still in place, insert it into a receiving slot in the PointCareNOW instrument for analysis, thus eliminating operator contact with blood. Results are available in 8 minutes. The PointCareNOW system can handle about 50 samples per

day. The system requires constant electricity and refrigeration. However, portable battery power and solar charge systems are available.

PointCare expects the new version of the device to provide a printed-out warning to clinicians and caregivers when patients have "out-of-range" hematology results and urgent clinical action is required.

This technology can be used at central, regional, district and some well-developed primary sites with dedicated laboratory facilities and technicians. It requires a moderate level of training (2-3 days).This system is FDA cleared and CE-IVD marked.

A previous multisite independent evaluation showed the suboptimal performance of this platform. However, further evaluations may be required to determine the efficacy of this platform for CD4 enumeration for the clinical management of HIV.

CyFlow® CD4 miniPOC

Partec has introduced a compact, portable CD4 counter that uses flow cytometry (including laser modules, optics, fluidics and electronics) to provide CD4+ T-cell and CD4 percentage enumeration. The device uses dry CD4 reagents, eliminating the need for cold chain or cold storage. This platform can run up to 250 CD4 tests per day, but can also be used in small health centers and other sites with a lower daily volume of testing. The CD4 miniPOC requires only 20µL of blood. The blood is added to a reagent-filled tube and incubated for 15 minutes. Buffer is added, and ultimately the sample blood is drawn up into a syringe to a precise fill line. The operator then places that syringe onto the POC device and the instrument slowly injects the processed sample into the instrument where CD4 detection takes place. Sample processing is not automated and takes place outside of the device. This system is CE IVD marked. This system can be used at many levels in the healthcare system including central, regional, district and mobile labs, as well as in some well-developed primary sites with dedicated laboratory facilities and technicians. It requires a moderate level of training (2-3 days). To date, no peer-reviewed, independent performance evaluations of the Partec CD4 miniPOC device have been found in a literature review.

Daktari™ CD4 Counter

Daktari Diagnostics, Inc. is developing a portable CD4 device, (Figure 4.5) with its associated cartridge. It is a small, portable device intended for use at POCs. It uses a cartridge microfluidic-based system to selectively capture CD4 cells in whole blood and to count them by electrical sensing.

Currently, the product can only do absolute CD4 counting. However it is expected to be capable of other assays, which may include full blood counts, CD4 percentages, and bacterial and viral

diagnostics.

No manual sample preparation or pipetting is required. The protocol is to lance finger, apply blood drop to cartridge, insert into CD4 counter, press “start,” and finally read result from LCD screen or printout. Venipuncture blood may also be used via capillary tube transfer. The dried reagents require no refrigeration and are shown to be stable up to 50° C in preliminary studies.

The Daktari instrument reports the CD4 count in less than 10 minutes. The CD4 system has not yet been launched, but performance evaluations are underway with market launch expected in the near future. There is currently no published performance data available for the Daktari CD4 system.

MBio POC CD4+ T-cell Counting System: MBio Diagnostics, Inc. is coming out with a POC CD4+ T-cell counting system that utilizes disposable cartridges and a simple reader instrument. It is designed for use in peripheral labs, clinics, and at POCs. The instrument functions as a two color fluorescence imaging cytometer and delivers absolute CD4 counts based on immunostaining and direct cell counting. The first cartridge product will deliver an absolute CD4 count, with following cartridges providing CD4 percentages and hemoglobin on the same system. Pipeline products include immunoassays for HIV and opportunistic infections such as syphilis, viral hepatitis and tuberculosis.

Cartridges can be processed in parallel (batch mode), using a separate cartridge rack with automatic timing. Turnaround time for a single sample is 20 minutes. Finger-stick or venipuncture whole blood samples are processed on the cartridge and then inserted into the instrument for reading, providing a throughput of over 10 samples per hour. The high sample throughput will allow healthcare settings with large POC CD4 testing requirements to meet their demand with fewer instruments. Blood and assay fluids stay on the sealed device, minimizing biohazard handling. Pre-market field evaluations in sub-Saharan Africa were initiated in 2012.In-country clinical trials are scheduled for the second half of 2013. A CE-IVD mark on the MBio CD4 System is anticipated in 2013 followed by product launch. This platform, though not currently on the market, is expected to be available in the near future.

- **Zyomyx CD4 platform**

Zyomyx, Inc. has developed a quantitative CD4 readout in a device free POC format. The system consists of a cartridge along with a small, mechanical mixer/spinner used in the test procedure. Inside the cartridge, the CD4 cells of a given blood sample specifically bind to heavy, anti-CD4 antibody coated particles. The cartridge is subsequently spun slowly in the mixer/spinner whereby only the conjugated cells penetrate into a high-density medium, forming a cell stack width in a small micro-capillary. The CD4+ T-cell count is proportional to the stacking height of the cells in that capillary, which can be visually read without the need of an electronic reader.

Minimal training is required. The primary skill required is the ability to perform a correct lancet blood draw. This system can be used at all levels of the health system (including health centers, mobile facilities, or in the field).

There is currently no performance data available for the Zyomyx system; clinical trials are underway. This platform, though not currently on the market, is expected to be available in the near future.

Visitect CD4 (Burnet Institute and Omega Diagnostics Ltd.): Burnet Institute (Burnet) has licensed its CD4 technology to Omega Diagnostics Ltd. (UK). The platform, which is now called the Visitect CD4, is a rapid, disposable semi-quantitative CD4 test. This system comprises a disposable cartridge containing a test strip (lateral flow) that measures CD4 proteins on T cells qualitatively (above and below 350 cells/µl).

The approach of the test is to measure CD4 proteins on T-cells, rather than to directly measure CD4 cells. Since the amount of CD4 per CD4+ T cell is constant throughout HIV, the total cell-associated CD4 should correlate with the CD4+ T-cell count.

The Visitect CD4 test has been incorporated into a lateral flow strip (similar to an HIV rapid diagnostic test) with a traditional rapid test format, including monocyte removal pad and immunogold conjugate. .Due to concerns about user's ability to read the test results, which requires users to identify the result line and compare it with the reference and controls lines on the strip, Burnet developed a reader for the device. It provides data storage and connectivity options, as well as real-time operating instructions for the test devices. The reader was developed in collaboration with Axxin Ltd. The test is disposable and does not require service/maintenance. The reader is expected to be robust and will be swapped out if it fails.

Clinical validation trials of the Visitect CD4 are underway. The Visitect CD4 is expected to be available for commercial release in the near future.

FACS Presto (BD Biosciences): BD Biosciences is developing an image-based counting technology called FACS Presto that will provide CD4 absolute count, CD4 percentage, and hemoglobin all on the same single-use disposable cartridge.

Features of the automated device include touch screen user interface, technician-friendly operation, flexible workflow with high throughput, integrated micro-printer, battery or solar-powered capability, and data archive/transfer capabilities. The sample is collected from the patient using a finger stick or an ethylenediaminetetraacetic acid (EDTA) tube. The cartridge is self-contained and is inserted by the operator into the device. After a short incubation period detection takes place automatically and the result can be read immediately in a single, easy

step. The cartridge technology contains dried reagents and requires no-cold chain, which enables longer shelf-life over a wide range of environmental conditions. There is currently no performance data available for the BD FACS Presto system; clinical evaluations are expected to complete shortly.

Variation in absolute CD4 counts: The CD4 counts obtained by different methodologies or equipment could vary from 10% to 30%. Proper implementation of quality control measures can reduce this variation to as much as 10%.

Figure 4.6: POC CD4 Device from BD Biosciences

Table 4.1: Available Methodologies for CD4 + T- lymphocyte enumeration

Operating principle	Capacity	Description of processing	Model name & manufacturer	Current Cost per test US $	Results
Standard Flow Cytometry based methods					
Flow	High throughput; 250–350 tests/day;	Flow rate with beads	FACSCalibur (Becton Dickinson)	3-6	Absolute CD4 count and percentage
Flow	High throughput; 250–350 tests/day	Flow rate with beads	EPICS XL (Beckman Coulter)	2-4	Absolute CD4 count and percentage
Flow	Mid to low throughput; <50 tests/day	Volumetric	CyFlow SL 3	2-4	Absolute CD4 count and percentage
Modified flow based methods					
Flow	Low–intermediate throughput; 30–100 tests/day;	Universal volumetric	Cyflow Counter (Partec)	8-10	Absolute CD4 count
Flow	Low–intermediate throughput; 30–100 tests/day;	Dedicated flow rate with beads	FACSCount, (Becton Dickinson)	4-10	Absolute CD4 count
Flow (micro capillary)	Low–intermediate throughput; 30–100 tests/day;	Dedicated volumetric	Guava easy CD4 (Guava)	1-3	Absolute CD4 count and percentage
Point of Care assays					
Flow	20 tests/day	Fixed volume fluorescence imaging	PIMA Analyzer (Alere)	2.5-10	Absolute CD4 count
Light scattering	50 tests/day	Forward light scattering and colloidal gold labeling	PointCareNOW (PointCare Technologies)	8-10	Absolute CD4 count and percentages, WBC count, haemoglobin, TLC and differential WBC count and percentages
Flow	250 tests/day	Volumetric flow cytometry	CyFlow™ CD4 miniPOC (Partec GmbH)	2.5-10	Absolute CD4 count and percentages

Criteria for Selection of the CD4 enumerating equipment:

For efficient and optimum reporting of CD4+ T cell counts the proper selection of the equipment is essential. The following points should be considered while selecting the equipment of a given laboratory

- Purpose of the assay (whether it is being used for monitoring or for research)
- The age group of the patients (whether adult or paediatric: to indicate whether CD4 percentages or absolute counts are required)
- Number of specimens to be tested/day
- Availability of stabilized electric power supply and space
- Location of testing (whether rural or urban and whether at primary health centre, district or central referral centre)
- Availability of technically skilled personnel as required (the current methods require varying degrees of technical skills)
- Availability of technical support for the equipment (regular maintenance is necessary)
- Cost: The cost should include equipment, reagent, consumable and maintenance costs.
- The turnaround time

While manufacturers test technologies/equipments extensively, an additional validation process, using established methods and instrumentation, is highly recommended. The equipments should be carefully reviewed for the technologies used and the needs of the laboratory considered when making procurement decisions. The equipments that have not been adequately validated should not be purchased.

References:

1. A Quality Management Systems Approach for CD4 Testing in Resource-Poor Settings: Am J Clin Pathol 2010; 134:556-567
2. Performance of the PointCare NOW System for CD4 Counting in HIV Patients Based on Five Independent Evaluations:Bergeron et al. PLoS One 2012

Chapter 5

Safety in CD4 laboratories

It is important for all personnel dealing with blood collection, transport, processing & examination to be protected from accidental exposure to blood and body fluids being handled. Accidental exposure to blood borne pathogens like Hepatitis B virus & HIV may occur, if standard safety precautions are not followed. Therefore, laboratory workers should be aware of the risks in their daily work and follow effective practices to prevent transmission.

In addition to biosafety, biosecurity, fire, chemical and electrical safety are all important aspects of total laboratory safety. These measures avoid laboratory accidents and reduce potential chemical, physical, and biological hazards. Any sample could be source of infection, which might result in morbidity or mortality if improperly handled; all samples should be treated as though they are potentially infectious. The standard safety precautions that must be followed while working with the biological samples should be known and understood by all the concerned staff.

This chapter deals with the important safety and biosafety measures for CD4 laboratories.

Biosafety: It is defined as the application of good laboratory practices and safety procedures, when working with potentially infectious microorganisms to protect the laboratory and other staff.

Biosecurity: Biosecurity is the application of measures that are addressed through the coordination of administrative, regulatory, and physical security procedures & practices implemented in a working environment for reducing the risks of biological material loss, theft, or misuse caused by poor management or poor accountability and protection.

Personal Protective Equipment: Personal protective equipment used in the laboratory include laboratory coats, protective eyewear, shoe covers, masks, and gloves. The laboratory coat and the gloves must be worn when handling potentially biological materials.

Additionally, every laboratory must have an easily accessible and well-labeled first aid box. The first aid box must contain all the emergency medicines needed for cuts, burns, etc. The label on the box must specify the contents of the box for easy reference. The first aid box must be checked regularly and any material that has been used up/expired must be replenished to ensure that the box has all essential supplies at all times.

Bio-waste segregation and disposal:

As per the Bio-Medical Waste (Management and Handling) Rules, 2011, segregation should be done at the source using color coded leak resistant bags for all solid contaminated bio waste

(Refer National Guidelines on Quality Management Systems In HIV Testing Laboratories). The bags should be tied tightly after they are three-fourths full and disposed off as per the prevailing State pollution control biomedical waste regulation (management and handling) rules 2011 guidelines.

Disinfection of the Work Surface: Work surfaces and equipment should be cleaned with 70% alcohol before and after completion of the laboratory's daily work.

Disinfection of the Flow Cytometer: Disinfect the flow cytometer as recommended by the manufacturer. One method is to flush the flow cytometer fluidic chambers with a 1% 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.

Disinfect flow cytometer waste by adding waste materials to the waste container; add a sufficient volume of 1% sodium hypochlorite.

Management of Spillage of potentially infectious material: The spills should be disinfected using sodium hypochlorite solution. In case of an infectious spill or blood spill, the spill should covered with absorbent paper/ cotton. Proper care should be taken if it contains glass pieces. A 1% Sodium hypochlorite solution should poured on the spill area and allowed to remain there for half an hour. The spill should be then cleaned wearing double gloves. All contaminated materials should be disposed of properly and carefully and the event must be documented.

Discarding Samples: The specimen collection tubes (evacuated blood collection tubes) should be decontaminated by autoclaving and disposing them as biomedical plastic waste as per the prevailing State pollution control biomedical waste regulation (management and handling) rules 2011 guidelines.

Discard jars should be made of polypropylene, which are robust and autoclavable. They should be 3/4 filled with freshly prepared 1% sodium hypochlorite solution every day. The jars should be emptied every day. The items to be discarded in the jar include used micropipette tips and tubes used for processing the specimens. Before discarding the materials in the jars, check that all the discarded material is properly submerged in the hypochlorite solution.

Standard Work Precautions: Standard work precautions (universal precautions) should be observed with all specimens received in the laboratory. The following safety practices must be followed:

- Wear laboratory coats, shoes, and gloves when processing and analyzing specimens, including working on CD4 equipment.
- Never pipette by mouth. Use safety pipetting devices.

- Never recap needles. Dispose of the needles and syringes in puncture-proof containers.
- Handle the specimens and other processes (e.g., aliquoting, adding reagents, vortexing, and aspirating) using Personal Protective Equipment (PPE) or in class II biological safety cabinets where available.
- Centrifuge specimens ensuring that the lid of the centrifuge is firmly fastened.
- After working with specimens, remove gloves; discard them according to the existing local biomedical waste management and handling rules and wash hands with soap and water.
- Follow the manufacturer's recommended procedures to eliminate the operator's exposure to any aerosols or droplets from specimens.

Post Exposure Prophylaxis (PEP): Any exposure should be immediately reported to the laboratory In-charge. The action should be taken as stipulated in National Guidelines for HIV Testing chapter 9 on 'Occupational Exposure and Post Exposure prophylaxis'. For further details refer to National Antiretroviral Therapy Technical guidelines 2013

Fire Safety Measures: Fire extinguishers are the most important safety equipment that needs to be provided in the laboratory. A portable fire extinguisher is the first line defense against small fires. It should be important to identify and place only suitable fire extinguishers in the laboratory. The laboratory personnel must be trained to use the fire extinguisher as a part of the safety drill training. Regular maintenance and servicing of the fire extinguishers must be done as per the manufacturer's recommendations.

A fire alarm system in the laboratory is essential. Standard operating procedures (SOPs) for fire alarm systems should be developed for fire accident prevention. The locations of the fire safety devices should be mentioned in this SOP.

The staff must be trained on escape routes (evacuation plans) in case of fire. All staff must practice fire drills so that they are well equipped and trained to respond in case of accidental fires. In case of fire, the staff must:

Do's:

- Leave the area immediately.
- Follow the procedures mentioned in the SOP.
- In case someone's clothing catches fire, the person should immediately stop, drop to the ground, and roll in an effort to extinguish the fire. If possible, remove the article of clothing before the skin catches fire.

Don'ts:

- Don't use the elevators.
- Don't extinguish fire in the laboratory with water because most laboratory fires are electrical

or chemical and water will generally be ineffective and spread the fire.

Electrical Safety

Electrical Safety of Equipment: The flowcytometer requires one dedicated circuit. Other laboratory equipment (e.g. the computer, refrigerator) requires a separate circuit. Operating other equipment on the same electrical circuit may cause intermittent failures, resulting in loss of data or component failures.

Electrical safety measures:

Equipment working on electricity have following requirements:

- Proper voltage regulators/UPS, and surge protector.
- Electrical equipment wiring, earthing, fuses, and switches should be checked regularly. An electrical safety check label is pasted on all equipment after the electrical engineer performs the electrical safety check. This should be done every three months.
- Electrical equipment must not be handled when in contact with wet surfaces or floors.
- Avoid the use of extension wires.
- Pest control for preventing damage to electric circuits in flow-cytometer is strongly recommended.
- Any obvious damage or defects must be reported to the supervisor/Safety officer and the maintenance department for proper action. It is strictly forbidden to use equipment when in doubt. Equipment should be clearly labeled with the following tag if it is found to be defective.

Chemical Safety

All chemicals and reagents must be clearly labeled with date of preparation and date of expiry. These must be properly stored with respect to heat, light, and proximity to other chemicals. Whenever chemicals come in contact with skin, the area must be cleaned thoroughly with soap and water.

Material Safety Data Sheet (MSDS): MSDS is a chemical hazard control form provided by the manufacturer. This includes basic information on rodent identification, hazardous ingredients, physical and chemical properties, fire and explosion hazard data, stability and reactivity data, health hazard data, precautions for safe handling and use, and special control measures. Any chemical, which is present in the laboratory must have an MSDS sheet from the specific manufacturer of that chemical. The laboratory personnel must read and understand the MSDS of the chemicals being used in their laboratory and file them properly for easy referral when needed.

Eyewashes: Eyewash stations are important to have in case harmful chemicals or biological

specimens splash into the eyes. Eye washing stations must be installed in every laboratory. It is recommended that eyes be washed with water for at least 15 minutes if there is contamination of eye by harmful chemicals. An eye washing station should be located near the workplace and be free from dust and dirt, easy to operate, easy to reach, and must be kept clean at all times. Maintenance and servicing of the eye washer should be done regularly.

Note: Safety is important in the laboratory and the staff should be thoroughly trained on all SOPs related to safety.

Specimen collection and transportation

Proper collection and transport of the specimen is important for quality CD4 results. For most of the methodologies, blood specimens collected in an appropriate container should be tested within 30 hours of collection. Specimens are stored at 20° to 25°C temperature, in a horizontal position, in the dark, and care must be taken to avoid vibrations/jerks. This minimizes cell loss during storage and keeps the cells in their native state.

Pre-analytical Considerations

1. Blood specimens should be collected from the same person at similar times of the day to avoid variations in the results due to diurnal fluctuations.
2. A sample should be collected using an evacuated blood collection system containing anticoagulant. The use of a syringe should be avoided to minimize the risk of needle stick injury and hemolysis.

Materials Required for Collecting Blood Samples:

1 PPE
2. K3/K2 EDTA evacuated blood collection tubes and needles.
3. Spirit / 70% alcohol
4. Cotton swabs
5. Tourniquet labels
6. Sample transport box
7. Cool packs (whenever required to keep the temperature at an ambient: 20° to 25°C)
8. Discarding jar containing 1% Hypochlorite
9. Needle destroyer
10. CD4 Cell Count Request and Report Form

Sample Collection and Handling:

1. The tube should be labeled with patient's identification, date and time of collection after checking for the expiry date. The expired tubes should NOT be used.
2. A full blood draw should be collected in a K3 or K2 EDTA evacuated tube (depending upon tube capacity, 3-5ml) using the standard procedure for venipuncture blood collection. Inadequate volume in a higher capacity tube is not recommended, as the anticoagulant is hypotonic and can affect the accuracy of the results.
3. The blood should be mixed properly; the tube should be inverted 6-8 times immediately after collection since the formation of small clots might affect the accuracy of the counts and lead to the clogging of the instrument tubing.
4. The tube labels should be verified to ensure that they match correctly with the test requisition form before sending it to the testing lab.
5. The requisition form should be filled with the age and sex of the patient, the collector's

information (name and initials), and other relevant details.

6. The specimens should reach the testing laboratory within 30 hours of specimen collection or as per the requirement of CD4 technology being used in the CD4 laboratory.
7. The specimens should not be refrigerated or frozen but should be stored at an ambient temperature (20-25°C). In case of very high temperature in summer, a cool pack should be used to maintain an ambient temperature. Temperatures greater than 37 °C may cause cellular destruction and affect both hematology and flow cytometry results.
8. Before transporting to the testing laboratory, the tubes should be examined for specimen integrity, e.g., whether the sample has clots.
9. Specimens should be processed within a maximum of 48 hours of collection for FACS Count and FACSCalibur. As per the guidelines from Partec, Specimens should be processed within a maximum of 6 hours of blood collection or else the blood can be stored at 2-8°C for 48 hours

Packaging and Transportation of CD4 Specimens:

Within the premises of collection center the specimens should be transported at an ambient temperature (without exposure to high temperatures) along with the CD4 Count Request and Result Form –Annexure 9)

If the samples are to be sent out station,

The packaging and transportation of HIV-infected material should be in accordance with the International Air Transport Association (IATA) regulations. The IATA and Transportation of Dangerous Goods Act regulate the shipping of specimens containing infectious agents. HIV is classified as an infectious class 6.2 substance under the United Nations (UN) no. 2814. Under proper labeling instructions, the packaging must adhere to UN class 6.2 specifications.)

Packaging Instructions of CD4 Specimens:

- The specimen should be carefully packed to protect it from breakage and insulated from extreme temperature.
- The caps of the vacutainers should be secured tightly and sealed with sticking tape in such a manner that it covers the lower part of the cap and some part of the stem.
- During packaging, the tubes containing specimens should be placed in a leak-proof container (e.g., a sealed plastic bag with a zip lock or, alternatively, the bag may be stapled and taped) or a tube rack and packed inside a cool box (thermocool or plastic) with cool gel packs (so that the temperature is maintained between 20° to 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. A cool box required for transportation could be a plastic breadbox or a vaccine carrier. Then seal/secure the lid of the cool box.
- This cool box should then be placed in a secure transport bag for the purpose of shipping it to the testing facility. The request slips should be placed in a zip lock bag and fastened securely to the outside of the box.

- A biohazard label should be pasted on the outer surface of the package containing the specimens. The package must be marked with arrows indicating the 'up' and 'down' side of the package.
- The specimens should be transported to the receiving laboratory by commercial courier or by hand delivery through a trained person following the transportation regulations.
- The collection site must have prior knowledge of the designated testing days of the laboratory to which the specimens are being sent.
- No transport should be done during weekends and holidays or non-testing days for the laboratory to which the samples are being sent unless the receiving laboratory has made prior arrangements.

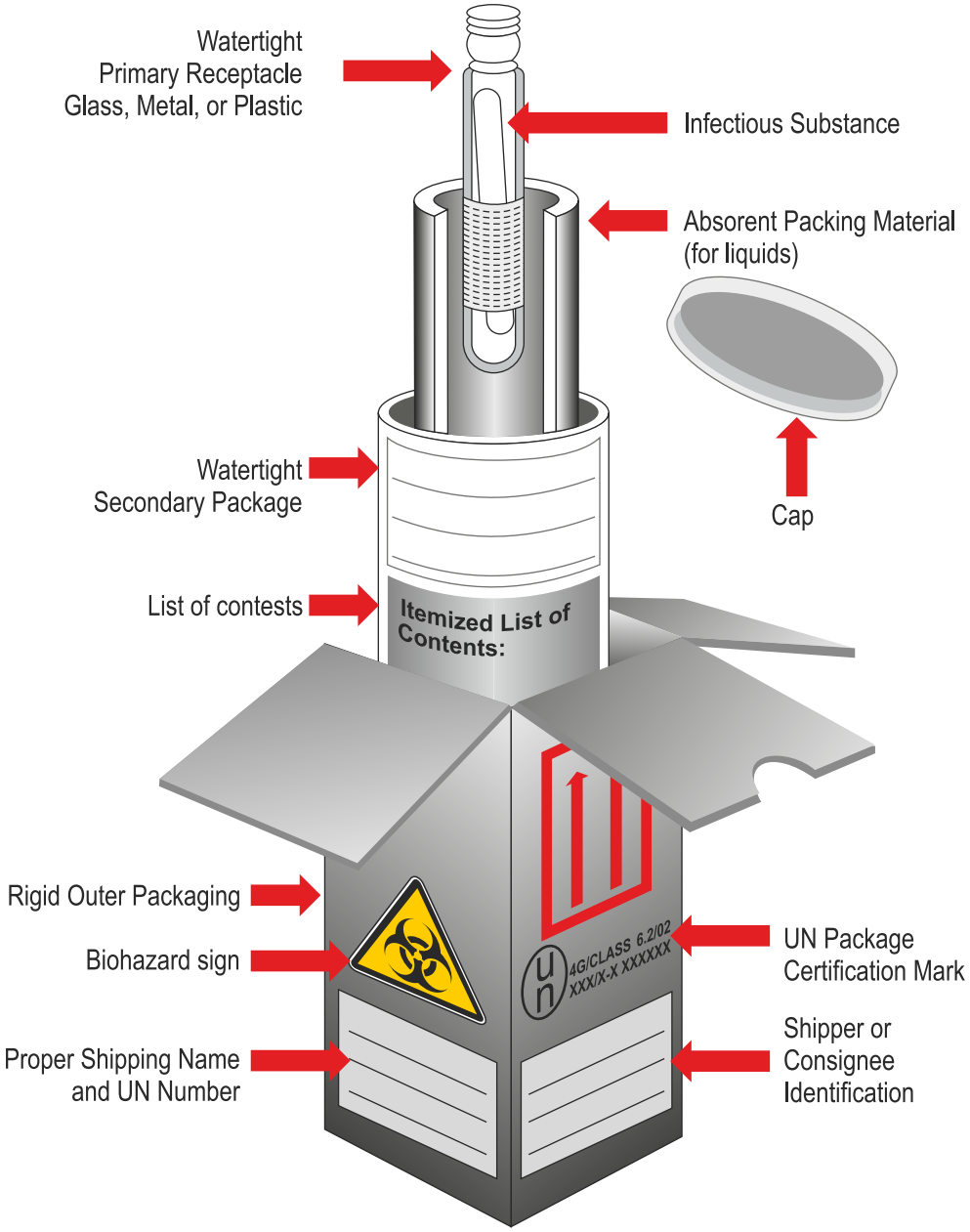


Figure 6.1: Packaging and labeling for the transport

Chapter 7

Quality Management

Quality management is essential for any laboratory, to ensure that the results are reliable, reproducible, traceable, and auditable. The CD4+ T cell count is an essential element of the management of HIV infected individuals. Therefore, ensuring quality control in the laboratory for the correct management of HIV infection is of the utmost importance.

Quality management incorporates the principles of quality control, quality assurance, and continuous quality improvement.

Quality Assurance (QA):
QA refers to all those overall planned and systematic activities that provide confidence in the fact that the results given out by the laboratory are correct. It is a program that is designed to monitor, evaluate, and improve the pre-analytical, analytical, and post-analytical processes in the laboratory to ensure the release of reliable results. It involves two components: Internal and external quality control (QC).

QC refers to the day-to-day operational techniques undertaken to ensure that the requirements for quality are met. It is a set of procedures undertaken by laboratory staff for the continuous monitoring of operations to ensure that the results are reliable & accurate enough to be released. The essential elements of the QC include personnel (i.e., laboratory staff), equipment, document control, reagent control, and corrective action towards problems encountered. QC should be used to monitor both sample processing and instrument performance within the laboratory. The External Quality Assessment (EQA) or external quality control includes participation in a proficiency program conducted by an external agency.

Key elements of Quality Assurance

1. **Personnel (staff):** It is important to have documentation of laboratory staff in an individual file. This should include qualifications, job responsibilities, trainings, and initial & periodic competency assessments
2. **Training:** Training is an integral part of quality control. The laboratory staff must be trained in the work assigned to him/her. Proper documentation of completed trainings should be kept for each staff member. Training in other areas related to work is desirable.
3. **Standard Operating Procedures:** The SOPs for every procedure carried out in the laboratories should be prepared and available for the person carrying out that procedure. The SOP should outline the procedure in detail and should be written by the person who carries out the procedure. It should be then reviewed and approved by the in-charge. The SOPs should be reviewed annually and modified if necessary. Annexure II provides the list of SOPs required in the CD4 enumerating laboratory.

4. **Use of Internal Control in the CD4 Count Enumeration:** The internal controls (stabilized blood samples or the fresh blood samples with known CD4 counts) should be included with every testing protocol from staining, acquisition and obtaining of the CD4 counts. The SOP should be prepared and followed to use these controls in daily testing.

At least two levels of internal controls- normal and low, should be tested in each run. If only one control is available it should cover the medical decision limit i.e. CD4 count ~ 350/mm3. Controls must be treated in the same way as patient samples and must be analysed in each batch/run. The test is valid when the controls produce acceptable results. Controls must be included in each run.

Types of internal quality control:

- Commercial: provided by manufacturer and cover normal and low levels.
- In-house controls: An alternate to commercially available internal controls is use of retained samples from HIV-positive or negative individuals.
- The values of in-house controls can be monitored by comparing with values on previous run (not more than 48 hrs). % difference of ≤15% is acceptable. Whenever an IQC failure is observed it should be reviewed & corrective actions taken as defined in the QMS.

The values of commercial controls can be monitored and reviewed on Levy Jennings (LJ) chart for Shifts and Trends. Various point of Care CD4 machines have in built system of internal quality control . If so, manufacturer's instructions should be followed.

- Shift indicates abrupt change in control mean. This indicates a major change in test performance due to 1) change in lot, 2) new reagent, 3) change in temperature 4) change in pipettes 5) a new technician, etc.
- Trend is a sustained increase or decrease in a quality control value over a period of time. Causes of trends are 1) gradual deposition of debris in tubings, 2) ageing of reagents, 3) gradual deterioration of control/calibration, etc.

For more details related to Quality control, LJ charts, use of Multirules, etc. refer to National Guideline on QMS in HIV Testing Laboratoriess.

5. **Lot-to-Lot Variation:** Whenever a new lot of reagents is received in the laboratory, it should be checked for reproducibility with the current lot. This should be done well in advance when enough stock is on hand. The percent variation should certainly be below 10 percent, however, less than 6 percent is desirable.

6. **Reporting of Results:** It is important for the laboratory to keep the source document, i.e. the equipment generated report, in the laboratory. The reports for the CD4 counts should be given in a prescribed format. This report form should include information on the patient ID,

name of the equipment used, and the Biological reference values.
Note: If more than one instrument is used in the lab for the same test, verify that results are concordant / equivalent and this should be done at least twice in a year.

7. **Data Backup:** For safety, backup of the data should be stored in a place other than the work place. An electronic back up should be made on CDs or hard drive. Thermal prints from the machine should be photocopied. The equipment generated printouts /result sheets should be stored in a safe place protected from accidents (e.g., fire) for a period of 5 years or more if so recommended by the local institution. If possible, efforts should be made to store the data electronically by entering the data into the database. The backup of such electronic data should be generated. One copy must be kept in the lab and another with the Head of the Department/in charge.
8. **Equipment:** For proper equipment function, it is essential that the equipment be properly maintained. This can be achieved by daily and regular preventive maintenance. Daily maintenance of the equipment includes start-up and shut down procedures as per the SOP. Additionally, the laboratory should have an annual maintenance contract with the supplier, and the schedule should be strictly followed. The equipment should be also checked for electrical safety periodically.

The equipment files should be maintained in every laboratory which should include:

a) Identification of the equipment
b) Manufacturers name and serial number
c) Current location
d) Manufacturer's contact person & telephone number
e) Manufacturer's instructions, if available or reference to their location within the laboratory premises.
f) Maintenance (Maintenance plan daily, weekly, monthly) and calibration details
g) Details of Annual or Comprehensive Maintenance Contract (AMC/CMC).

Calibration and performance check on the Equipments:

To ensure that all test equipments continuously produce accurate & precise data, equipment calibration needs to be performed, either in house or through external agencies, on a timely basis as manufacturer's instructions. The frequency of calibration depends on manufacturer recommendation or as required by accreditation bodies like NABL. Calibrators used should be traceable. Equipment that is moved (e.g., due to repairs) from its location should be recalibrated and verified before it is brought back into use. For external calibration, the equipment manufacturer's services could be utilized. F Flow cytometer should be calibrated once a year (or as recommended by manufacturer). Calibration verification for centrifuges and pipettes should be done at least every six months.

Table 7.1 provides calibration frequency details for the equipment recommended in a CD4 enumerating laboratory recalibrated. The equipment should be checked for electrical safety periodically.

Table 7.1: Frequency of calibration of the equipment in CD4 estimating laboratory

Name of equipment	In-house Calibration frequency		External calibration
	Daily before sample run	Biannually	
Multichannel Pipettes		✓	May be possible
FACSCount	✓		✓
FACSCalibur	✓		✓
Guava	✓		✓
Cyflow	✓		✓
Biosafety Cabinet	✓		✓

Each lab should have a documented policy defining the procedure for appropriate instrument function checks (performance verification) to be performed prior to running patient samples. Instrument function checks are ideally done by commercially available reference beads. These may differ depending upon type of instrument. Hence it is important to use vendor supplied beads and respective automated software systems for monitoring the instrument. The parameters that may be monitored are- laser current and laser power, PMT voltages, fluorochrome sensitivity, laser delay (applicable on multi laser instruments), Window extension (when applicable), fluids and Area Scaling Factors (applicable on digital flow cytometers).

Frequency of performance check:
Laboratory shall have a policy on its instrument performance check based on its work load like daily, or after every cold start (when equipment was on power off mode and gets switched on). If instrument is not used regularly, it is recommended to do performance check at least once a week.

Compensation Controls: Compensation is an essential part of proper experimental setup for multicolor assays. Compensation may be done using microbeads (spherobeads) or cells containing mutually exclusive populations of the same fluorochrome. However; it is important to optimize the settings given by the beads with cells to be used in the actual experiment. Modern instruments have introduced capabilities for recording primary data simultaneously with the visualization of compensated data. Each laboratory needs to document the procedure of how the color compensation is going to be setup. This can be done manually or by automated methods. However, it is essential to re-establish compensation values after any hardware change, laser realignment, and change in filters, optics or any other such parameters which affect instrument performance. It is essential to note that compensation settings are stable for a

given set of PMT voltages. Change in PMT voltages may lead to adverse effect on compensation values, and should be avoided.

External Quality Assessment (EQA):

EQA is an evaluation of the performance of participating laboratories (e.g., accuracy) by an outside agency on specially supplied samples. The EQA helps in identifying problems in the testing and offers appropriate corrective measures to overcome the problems. Hence, the laboratories should participate in EQA. Under this program, the specimens are sent to the participating laboratories at two or four month intervals. For the CD4 count enumeration, the specimens are the stabilized blood specimens. These specimens are only stable for a specific period of time and, therefore, it is mandatory that these be tested as early as possible and not after the date mentioned on the form. It is also important to test these specimens as a routine patient's specimen. The external agency will analyze the results using statistical tools and assess the performance of the participating laboratory. After receipt of the performance report from the conducting agency, it is important to follow the corrective actions suggested by the agency. This improves the performance of the laboratory over time.

The Lab In-charge should, on receiving the PT results review them within stipulated period. The participating laboratory should take corrective action, where ever needed, depending on the results. The outcomes are communicated as per the reporting hierarchy.

Various international EQA programs are available, such as the Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS (QASI) from Canada, United Kingdom National External Quality Assessment Scheme (UKNEQAS) from the United Kingdom, or and the Center Of Excellence (COE) from Thailand. At the national level, NACO has developed EQA programs with the help of the National AIDS Research Institute (NARI). NARI conducts EQA for the CD4 laboratories under the NACO umbrella.

Safety and Archiving of Data

The laboratory should have controlled access to authorized personnel only; Laboratory data records (e.g., hard copies of the equipment printouts) should be stored in the lockable cupboard for the prescribed period stated in the institutional policies. Only authorized staff can handle the equipment and computers. Computers used for recording data must be password protected.

In laboratories, data and records are collected over a period of time. Eventually, it is necessary to back-up or archive the data for safety. The hard copies of the data can be scanned and stored electronically on external drives like hard disks/ pen drives/ CDs etc. The thermal prints generated from equipment need to be photocopied and then stored. If the datacan be

transferred on external drives (from equipment like FACSCalibur/Guava/Cyflow etc) then it can be stored as an e-copy on CD/USB drives. The backup of the data must be stored in a different location from the laboratory.

Audits: In order to verify that operations continue to comply with the requirements of the quality management system, audits for all elements of the system, both managerial and technical, are conducted at periodic intervals. The purpose of an audit is to ensure high quality procedural compliance, activities, and documentation, as per the quality system.

An audit may be categorized as internal and external.

Internal Audit: first-party audits, are conducted by, or on behalf of, the organization itself for management review and other internal purposes, and may form the basis for an organization's declaration of conformity. (ISO 9000:2005 3.9.1)

External Audits: External audits are conducted by a third-party. Third party audits are carried out by external, independent auditing organizations (e.g., NABL, PPD) such as those providing certification of conformity to ISO 9001, ISO 15189.(ISO 9000:2005 3.9.1).

References:

1. Parallel Testing and Reagent Lot Validation – Guidelines from IQA, Pro40-06 Parallel Testing Version 2.0

Chapter 8

Operational Requirements of CD4 Laboratories

This chapter deals with two important aspects regarding CD4 count enumeration in the Indian laboratories. The first aspect is the requirement of the CD4 estimating laboratories to provide for an optimum work environment & an ideal laboratory floor plan; the second aspect covers the operational guidelines for the CD4 enumerating laboratories under the NACO umbrella.

1. Requirements of the CD4 Laboratories to Provide an Optimum Work Environment and Recommended Laboratory Floor Plan

Electrical requirements

1. Two 220 VAC, 50/ Hz (± 10%), 15 amp dedicated circuits.
2. same as under the electric point -
3. The cytometer/loader requires one circuit while other laboratory equipment (e.g. the computer, fridge) requires a separate circuit.
4. Uninterrupted power supply (UPS) unit is required

Laboratory Space

1. A normally filtered, air-conditioned environment with a maintained ambient temperature of between 16°C and 29°C is required.
2. An environment with excessive mechanical vibrations should be avoided for optimum performance of the equipment. Avoid selecting sites near heavy-duty mechanical equipment as floor vibrations may impact the performance of the equipment.

Laboratory Bench (See floor plan ahead)

1. A stable, well-supported and level worktable or counter space made of granite or other cleanable acid resistant laboratory surface is required.
2. Width: minimum 300 cm
3. Depth: minimum 80-90 cm
4. Height above floor: minimum 80-100 cm
5. Clearance above bench: minimum 150 cm

Walls and floor

1. Tiles for 1.0 – 1.5 meters above bench top
2. Washable gloss, semi-gloss or equivalent paint on walls
3. Washable and acid resistant tiles on floor

Sinks

1. A dedicated laboratory sink should be made available in the CD4 testing lab.
2. A hand-washing sink should be available in the testing lab or in adjacent rooms before exiting the overall laboratory area.

Lighting

1. Adequate lighting

Storage

1. Cold reagent storage: Laboratory refrigerator without no frost facility and sufficient space to store the reagents and samples. Freezer storage compartment is not required.
2. Consumable storage: Laboratory cupboards at room temperature
3. Results archive: Filing cabinet

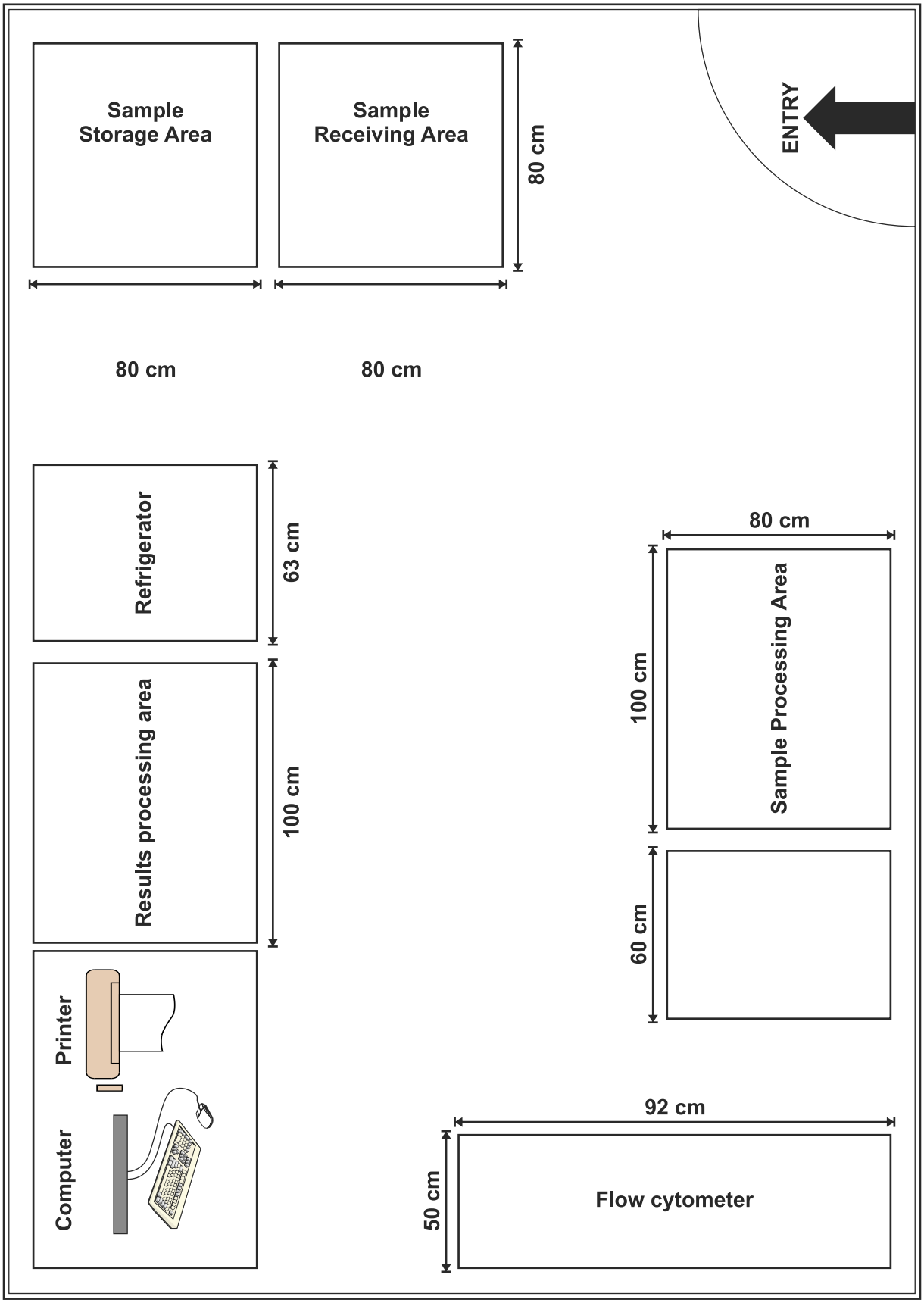
Waste Disposal

1. Minimum 3 floor standing waste disposal bins with lids
2. Bench-top discard jars/bins.

Other Lab Equipment / Consumables

1. Variable auto pipettes (10 µl, 20-200 µl and 100-1000ul)
2. Pipette tips
3. Vortex / cyclomixer
4. Blood rocker
5. Tube stands
6. K2/K3EDTA vacutainers
7. Sodium hypochlorite
8. Needle destroyer
9. Disposable gloves (powder less)

Suggested plan for CD4 enumerating laboratory



2. **Operational Flow of the CD4 Enumerating Laboratories under the NACO Umbrella**
   The CD4 centers are divided into nodal and linked centers, according to the number of patients attending ART clinics at that center.

Responsibilities of Nodal Centers

Nodal centers are those, which are placed at Medical college facilities or other facilities with trained laboratory supervisors that can maintain the technically intensive machines. These cater to a particular region or regions in the state as per geographical location. These centers are provided with the CD4 enumerating equipment. Their responsibilities are as follows:

In charge or Designee (signatory for the CD4 report): The In charges should be trained in CD4 count enumeration and quality control procedures in context with the equipment provided to the center. The other responsibilities include:

1. Training of the technicians and related documentation
2. Ensuring timely completion of laboratory records and other related documentation.
3. Review of all documents (pre analytical, analytical and post analytical)
4. Signing of the report
5. Monitoring of the quality control procedure and results of participation in EQA
6. Ensuring corrective action and trouble-shooting as per the requirements recommended by the EQA provider.

CD4 Technicians:

1. Carry out the procedures according to the SOPs
2. Carry out trouble-shooting and corrective actions as per the requirements
3. Completion of all documents
4. Participate in the GLP training
5. Help technicians from the linked center use the equipment for processing and getting CD4 counts from the samples brought in from linked centers.

Responsibilities of Linked Centers

CD4 Technician:

1. Communication with the nodal center for sample collection and processing
2. Collect and transportation of the samples to the nodal center on the decided day
3. Process the samples as per the SOPs
4. Completion of all documents
5. Transportation of the CD4 report back to the linked center
6. Timely hand over of the report to the patient
7. Participate in the annual GLP training.

The nodal centers are selected from within 200km of the linked centers and it is ensured that there is suitable transport to reach the nodal center within 24 hours of sample collection.

Transport and daily allowance (TA/DA) and handling charges are paid from ART center contingency funds where the technician is working. The networking is done with consultation and consent of the respective SACS.

Responsibilities of Training Center In-charge:

1. Arrange, preparing training materials and monitor the training
2. Provide information on the biology of HIV, laboratory safety, and related topics
3. Discuss and take up the issues of participants with NARI (technical), and SACS & NACO (administrative)

Training

NACO runs the quality control program for these centers. NARI, Pune helps NACO run this program for CD4 enumerating centers. The quality control program includes training in internal quality control, and participation in the EQA program.

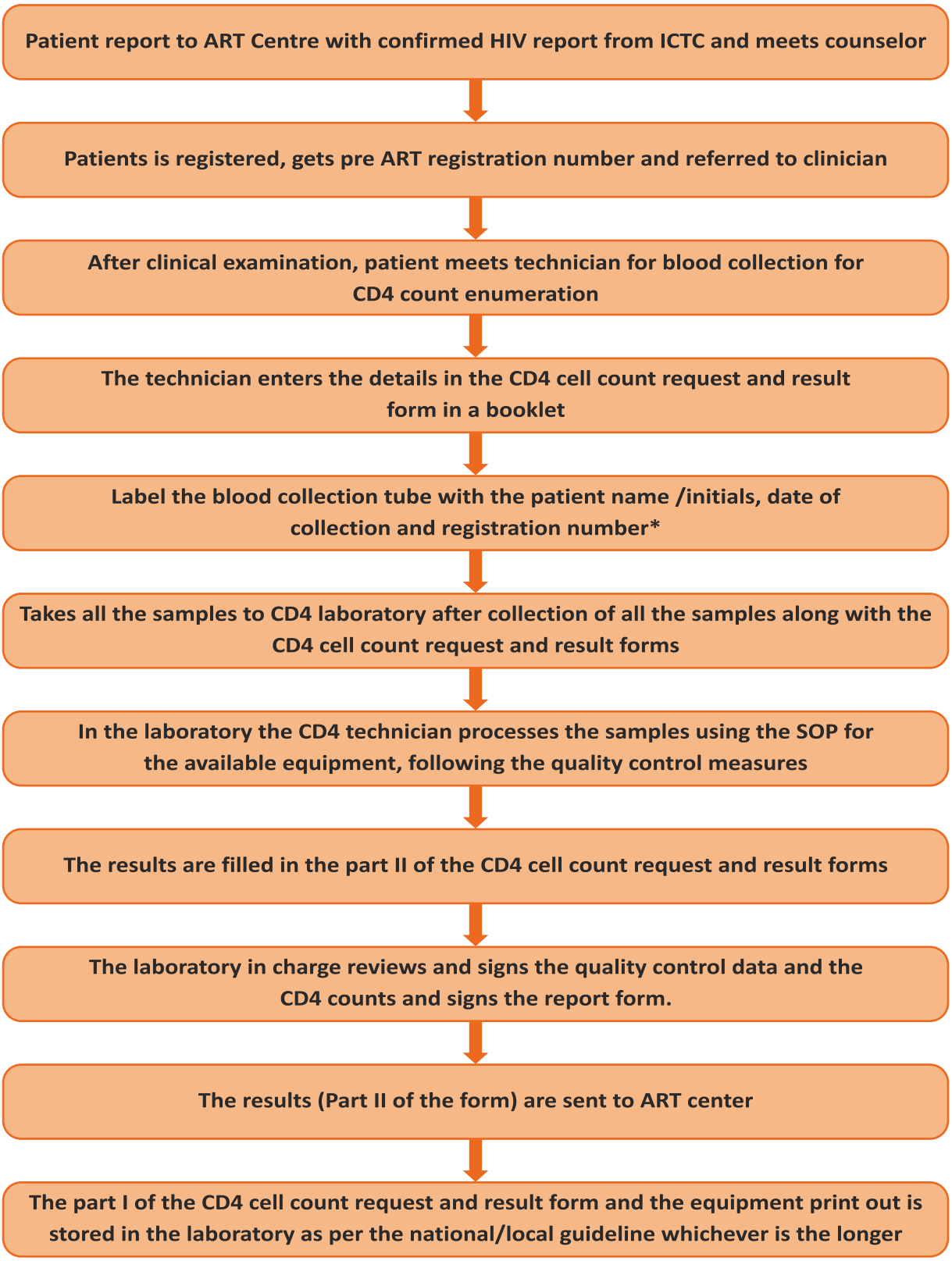
Trainings on Good Laboratory Practices in context with CD4 count enumeration are conducted annually at designated training centers. These training are equipment specific and are organized with the help of the manufacturers. The technicians from all nodal and linked centers participate in these training. The two/three day training is arranged to give training on equipment procedures, trouble-shooting, explaining the internal quality control, and EQAP. Additionally the participants are provided with the necessary information on HIV biology and the importance of HIV diagnostic and monitoring tests.

EQA for CD4 count enumeration:

All the nodal centers are participating in the EQA three times annually. The program is run by NARI, Pune. The stabilized blood samples are distributed to all centers. The centers process the samples as per the routine procedure and send the results to NARI. NARI analyzes the results of the individual laboratory in comparison with the mean of CD4 values from all laboratories and assess the performance of the laboratory. NARI also provides trouble-shooting for centers with unsatisfactory performance. It is important to participate in EQA and to address trouble-shooting issues to improve quality on an ongoing basis. It is also important to follow the SOPs for quality management for continuous overall performance improvement.

The laboratories must be proud to follow all the quality control guidelines and provide accurate and reproducible results.

Flow Chart for the procedure for CD4 count enumeration in ART centers



* The linked ART centers can collect the samples on the designated day and send it to the CD4 testing center with the technician.

DO's and DONT's in the laboratory

DO's

- Always wear a lab coat
- Wash your hands when you enter the lab, after finishing the work and before leaving the laboratory.
- Always clean up the working area after the work is over.
- Know where the fire extinguisher, eyewash station are placed and ensure that they are functional at all times.
- Refer to MSDS as applicable.
- ALWAYS wear gloves when dealing with chemicals and potentially infectious material.
- Wear eye shields when dealing with glassware and chemicals
- Follow safety procedures set in the laboratory.
- LABEL all the reagents with date of opening/preparation and expiry.
- **Always** take necessary volume of chemicals & **NEVER** put back chemicals into its container when you already took it out.
- Follow first expiry first out (FEFO) principle for usage of reagents and consumables.
- Wear shoes that are fully covering your feet.
- Report incidents of spills and occupational exposures to Lab-in-charge and maintain documentation of occurrence and action taken.

DON'T'S

- Store/eat food or drink in the laboratory
- **ASSUME!** Ask if you don't know or even unsure about little thing. Better **be safe**.

Annexure 2

List of SOP's required for the CD4 laboratory

This list includes the SOPs that are a must in the CD4 laboratory. However, the SOPs in an individual laboratory may not be limited to this list. Each laboratory must develop additional SOPs as per the requirement.

1) SOP specific to each Equipment including CD4 enumerating equipment such as FACSCount/FACSCalibur/Cyflow Partec/POC
2) SOP for Internal Quality Control Programme for CD4 count testing Laboratories
3) SOP for Pipette Calibration
4) SOP for Biosafety

SOP/CD4/01
Form 01

BD FACSCount Operational Log

Name of Laboratory: .....

Date	Name of user	Time (Start)	Start Up		Shut Down		Time Shut Down	Remarks	Sign
			Drain/Fill	Cleaning & Rinsing	Drain/Fill	Cleaning & Rinsing			

Supervisors Sign

BD FACSCount Reagent Log

SOP/CD4/01
Form 02

Name of Laboratory: .....

Date	Reagent Lot #	Reagent Exp Dt.	Control Lot #	Control Exp Dt.	Control Count, beads/ml				Control P/F	Sheath Lot #	Sheath Exp. Dt.	Comments	Operator's Sign
					Zero	Low	Medium	High					

Control Lot # Reagent Lot #

Zero Low Medium High (Beads/ml) Cd4 tube (Beads/ml)

Supervisors Sign

SOP/CD4/01
Form 03

BD FACSCount Monthly Cleaning Log

Name of Laboratory: .....

Date of Cleaning	Next Due Date	Name of Operator	Sign

Supervisors Sign

SOP/CD4/02
Form 01

BD FACSCalibur Operational Log

Name of Laboratory: .....

Date	Name of User	Time Started	Start Up		Shut Down			Time Shut Down	Remarks	Sign
			Priming 3 times	Cleaning Hypo &D/W	Cleaning Hypo	Cleaning D/W	Depressurize			

Supervisors Sign

SOP/CD4/02
Form 02

BD FACSCount Monthly Cleaning Log

Name of Laboratory: .....

Date of Cleaning	Next Due Date	Name of Operator	Sign	Counter Sign

Supervisors Sign

SOP/CD4/03
Form 02

PARTEC Cyflow Reagent Log

Name of Laboratory: .....

Date	Reagent Lot #	Reagent Exp Dt.	Count check bead green (CCB green) Lot #	CCB green Exp. Dt.	Control P/F	Sheath Lot #	Sheath Exp. Dt.	Comments	Operator's Sign

Supervisors Sign

SOP/CD4/03
Form 03

PARTEC Cyflow Monthly Cleaning Log

Name of Laboratory: .....

Date of Cleaning	Next Due Date	Name of the Operator	Sign	Counter Sign

Supervisors Sign

SOP/CD4/04
Form 01

Competency Record for Newly Joined staff for CD4 Count Estimation

Name of Laboratory: .....

	Sr No.	Date	Ab CD3	% CD3	Ab CD4	% CD4
1-CS						
1-Trainee						
% Variation						
2-CS						
2-Trainee						
% Variation						
3-CS						
3-Trainee						
% Variation						
4-CS						
4-Trainee						
% Variation						
5-CS						
5-Trainee						
% Variation						
6-CS						
6-Trainee						
% Variation						
7-CS						
7-Trainee						
% Variation						
8-CS						
8-Trainee						
% Variation						
9-CS						
9-Trainee						
% Variation						
10-CS						
10-Trainee						
% Variation						

* C.S.-Competent staff: Instrument Used: Trainee:

Comment: **Supervisors:**

SOP/CD4/04
Form 02

Competency testing for CD4 Count Estimation

Name of the Staff: ..... Name of the laboratory: .....

Instrument used: ..... Date of Processing: .....

Sample No.: ..... Trial No.: .....

Parameters	Values obtained	EQA results		Remarks
		Mean	2 S.D.	
Cd3 Abs				
CD3%				
CD4 Abs				
CD4 %				
CD8 Abs				
CD8 %				

Sample No.: .....

Parameters	Values obtained	EQA results		Remarks
		Mean	2 S.D.	
Cd3 Abs				
CD3%				
CD4 Abs				
CD4 %				
CD8 Abs				
CD8 %				

Signature of Staff: ..... Date: .....

Comment: .....

Signature of supervisor: ..... Date: .....

SOP/CD4/04
Form 03

Log for establishing range for commercially available control

Name of Laboratory: .....

Control ID: ..... Instrument Used: .....

Lot No. : ..... Expiry Date: .....

Sr No.	Accession No.	Date	Ab CD3	% CD3	Ab CD4	% CD4	Remarks/Sign
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
Mean							
S.D.							
2S.D.							
Range							

Comment: **Supervisors:**

SOP/CD4/04
Form 04

Log for Fresh Quality Control samples run everyday

Name of Laboratory: .....

Machine used: FACS Count / FACS Calibur / PARTEC Cyflow counter

Sample ID	Date	Abs. CD3	% CD3	Abs. CD4	% CD4	Sign of LT	Remarks
% Variation							
% Variation							

$$\% \text{ Variation} = \left\{ \frac{\text{Observed} \times 100}{\text{Expected}} \right\} - 100$$

Supervisors Sign and Date

SOP/CD4/04
Form 05

Quality Control Log - At change of Lot

Name of Laboratory: .....

Machine used: FACS Count / PARTEC Cyflow Counter

Sr. No.	PID. No.	Dt. of processing	Reagent Name	Reagent Lot	Reagent Exp. Date	Abs. CD3	Abs. CD4	Abs. CD8	Remark	Tech. initials	Sign
% Variation											
% Variation = $\left\{\frac{\text{Observed x 100}}{\text{Expected}}\right\}$ -100 % Variation											

Supervisors Sign and Date

SOP/CD4/04
Form 06

Quality Control Log - At change of Lot

Name of Laboratory: .....

Machine used: FACS Calibur

Sr. No.	PID. No.	Dt. of processing	Reagent Name	Reagent Lot	Reagent Exp. Date	Abs. CD3	CD3 %	Abs. CD4	CD4 %	Remark	Technician
% Variation											
% Variation = $\left\{ \frac{\text{Observed} \times 100}{\text{Expected}} \right\}$ -100 % Variation											

Supervisors Sign and Date

SOP/CD4/02
Form 07

Back up of Electronic Data

Name of Laboratory: .....

Date	Identification of back-up drive	Type of data		Checking of previous data	Signature of LT
		CD4 data base	Cd4 Documents		

Supervisors Sign and Date

SOP/CD4/02
Form 08

Corrective Action and Preventive Action Form (CAPA)

Name of Laboratory: .....

Identification of Non-conformance **Signature**
Corrective Action Proposed **Signature of Supervisor / HOD**
Responsibility and timelines **Signature of Supervisor / HOD**
Corrective Acton Taken **Preventive Action taken** **Signature of Supervisor / HOD**

SOP/CD4/05
Form 01

Pipette Accuracy Verification Log

Name of Laboratory: .....

Pipette No./Lab ID: ..... Make / Model No.: .....

Pipette Range: .....µl Analytical (Electronic) Balance ID: .....

Date of Verification: ..... Next Verification Due Date: .....

Verification Performed by: ..... Room Temperature: .....

Sr. No.	Set At (Lowest)	Actual	Set At (Middle)	Actual	Set At (Highest)	Actual
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
Mean						

SOP/CD4/05
Form 02

CALCULATIONS

Weight of Water Dispensed = (Weight of Petri dish + Water) - Weight of empty Petri dish

Expected Weight = volume X Density factor (See Appendix I)

Mean water weight = $\frac{\text{Sum of water weights for all tests}}{\text{Number of tests}}$

Percent accuracy = $\frac{\text{Mean water Weight X 100}}{\text{Expected weight}}$

CALCULATIONS	µl	µl	µl
Temparature of D/W			
Absolute density of D/W			
Weight of Water dispensed			
Edxpected Weight			
Mean water weight			
Percentage Accuracy			

Comments: The percent accuracy is within the acceptable range / not within the acceptable range

Sing of Technician:

Verified by:

SOP/CD4/05
Form 03

Pipette Verification Label

Pipette ID / Lab:	
Date of Verification	
Done by:	
Due date of Verification	

SOP/CD4/05
Form 04

LIST OF PIPETTERS

Name of Laboratory: .....

No.	Pipette No.	Description	Serial No.	In Use Status	Verification date	Comments

CD4 COUNT REQUEST AND RESULT FORM

Section1: For ART center/clinic

Name of the clinic: .....

Patient name: ..... Age/Sex: .....

Patient ID (pre ART/ART registration no): ..... Date of Birth: .....

Date of collection (DD/MM/YY) ..... Time of Collection (hr: min): .....

Signature & Name of the person drawing blood: ..... Signature of nodal officer /designee .....

Section 2: For Laboratory

Name of the Laboratory: ..... Laboratory no: .....

Date of receipt (DD/MM/YY): ..... Time of receipt (hr:min): .....

Date of testing (DD/MM/YY): ..... Instrument used: .....

Time of reporting: .....

Results: .....

Parameter	Value	Adult Reference Range		Pediatrics Reference Range		
		Male	Female	Upto 1 yr	1-5 Yrs	5-16 yrs
Cd4 Absolute Count (Cells / µl)		381-1565	447-1846	400-5300	500-5500	300-2100
Cd4 Percent		25-49	27-54	17-68	23-50	25-53

REMARKS

Please correlate clinically.

Please collect fresh blood for repeat test as the sample processed shows.

a] Sample was rejected due to receipt after 30 hours of collection / clotted / hemolysed condition.

b] Abnormally low or high values.

c] Others……

Technician's Name & signature

Lab-In-Charge: Name& signature

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Dr. A. S. Rathore, DDG CST Division

Dr. Sunil Khaprde, DDG Basic Services and STI/RTI Sevices

Dr. Ashok Kumar, *Former* DDG Basic Services and STI/RTI Sevices

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