A microscopic image showing several CD4 T lymphocytes. The cells are stained, with a prominent purple-stained nucleus and a lighter cytoplasm. They are surrounded by other cells, some of which are smaller and more numerous.

Laboratory Guidelines for enumerating CD4 T Lymphocytes in the context of HIV/AIDS



**World Health
Organization**

Regional Office for South-East Asia
New Delhi

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Natural history of HIV infection

1.1 Magnitude of problem

The HIV/AIDS pandemic has severely affected health development and eroded improvements in life expectancy, particularly in countries with the highest prevalence of infection. Since the first cases of acquired immunodeficiency syndrome (AIDS) were reported in 1981, infection with human immunodeficiency virus (HIV) has grown to pandemic proportions, resulting in more than 65 million infections and 25 million deaths. At the end of 2006, an estimated 39.5 million people were living with HIV, with Sub-Saharan Africa carrying the highest burden.

In 2006, an estimated 4.3 million people became newly infected with HIV. More than 95% of these new infections are in low and middle income countries. Each day 11,000 persons become newly infected with the virus; of these, half are women and 40% are young people (15-24 years old). Of the estimated 37 million adults living with HIV worldwide, nearly 18 million are women.

At the end of 2006, there were an estimated 2.3 million children living with HIV. The increasing number of child deaths due to AIDS threatens to reverse many of the recent gains of child survival programmes. Moreover, the socio-economic impact of HIV/AIDS on children is profound. As their parents fall sick and die of AIDS, children undergo a long trail of painful experiences such as: economic hardship, withdrawal from school; lack of love, attention and affection; psychological distress, stigma, discrimination and isolation, and malnutrition and illness. Cumulatively, there are a total of 16 million children worldwide who have become orphans because their parents died of AIDS.

HIV burden in South-East Asia Region

The WHO South-East Asia (SEA) Region includes: Bangladesh, Bhutan, the Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, Thailand, and Timor-Leste. The overall adult HIV prevalence in south and south-east Asia is 0.7%, relatively much lower

than the 5.7% prevalence in sub-Saharan Africa. However, due to the large populations, even low HIV prevalence means that large numbers of people are living with HIV.

At the end of 2006, there were an estimated 7.2 million people living with HIV in SEA Region. This included 0.77 million new infections in 2006. Approximately 550,000 persons died of AIDS during 2006. Five countries - India, Thailand, Myanmar, Indonesia and Nepal account for a majority of the burden in this Region. Long-standing epidemics have left a huge burden of people living with HIV/AIDS (PLHA) who need prevention, care and treatment services. Table 1.1 provides the estimated number of PLHA in each country in the Region.

Table 1.1: HIV/AIDS Burden in South-East Asian countries, December 2005

| Country | Adult HIV Prevalence (%) | Estimated number of people Living with HIV |
|-------------|--------------------------|--|
| Bangladesh | <0.1 | 7 500 |
| Bhutan | <0.1 | < 500 |
| DPR Korea | n/a | n/a |
| Indonesia | 0.2 | 193 000* |
| India | 0.9 | 5 200 000** |
| Maldives | <0.1 | <100 |
| Myanmar | 1.3 | 339 000** |
| Nepal | 0.5 | 70 000 |
| Thailand | 1.4 | 541 000 |
| Sri Lanka | <0.1 | 5 000 |
| Timor-Leste | <0.1 | <100 |

*data for 2006

** among persons aged 15-49 years

Source: Country reports, National AIDS Programmes, Ministries of Health

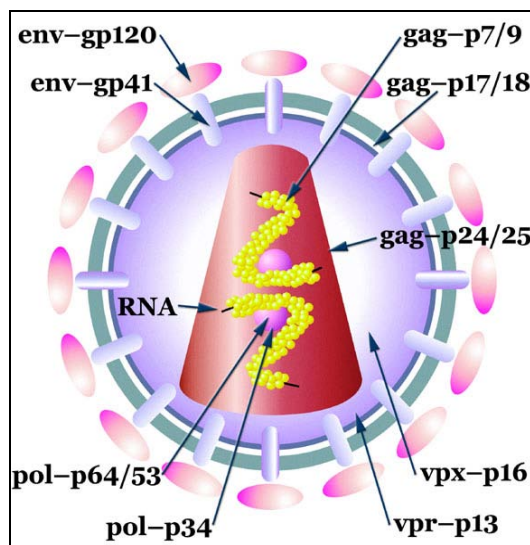
1.2 Biology of HIV

HIV is a lentivirus that belongs to the family Retroviridae and is characterized by single stranded RNA genome. It relies on enzyme reverse transcriptase for reverse transcription of RNA into DNA that gets integrated into host genome as a provirus.

Structure of HIV

HIV is an enveloped virus measuring about 120 nm in diameter. The virus core consists of two copies of positive single-stranded RNA that code for the nine genes enclosed in a conical capsid composed of 2,000 copies of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by the viral envelope which is composed of two phospholipid layers drawn from the host cell membrane when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrude through the surface of the virus particle. This protein, known as *Env* protein, consists of a cap made of three molecules called glycoprotein (gp 120) and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle. (Fig 1.1)

Figure 1.1: **Structure of HIV**

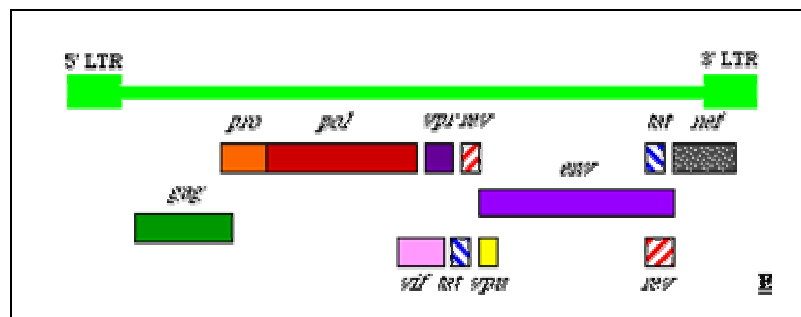


HIV genome

HIV genome carries nine genes and one long terminal repeat region at either end of the genome.

- Three structural genes, *gag*, *pol*, and *env*, code for information needed to make the structural proteins for new virus particles including Env protein, capsid and matrix proteins and enzymes integrase, protease and reverse transcriptase.
- The six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are regulatory/accessory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease. The protein encoded by *nef*, for instance, appears necessary for the virus to replicate efficiently, and the *vpu*-encoded protein influences the release of new virus particles from infected cells.
- The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell (Fig 1.2).

Figure 1.2: **HIV genome**



HIV life cycle

- **Binding and Fusion:** HIV uses CD4 molecules on the surface of the lymphocytes as a primary receptor. Viral gp 120 binds the CD4 molecule on the surface of CD4 T lymphocyte leading to

the conformational changes exposing binding site for coreceptors (chemokine receptors) present on the surface of a CD4 T lymphocyte. The virus envelope fuses with host cell membrane leading to the release of viral RNA copies in the protoplasm of host cell.

- *Reverse Transcription:* Viral reverse transcriptase transcribes the single- stranded HIV RNA to double-stranded HIV DNA that subsequently moves to the nucleus.
- *Integration:* An HIV enzyme called “integrase” integrates the HIV DNA to the host cell's own DNA. The integrated HIV DNA is called provirus. The provirus may remain inactive for several years, producing few or no new copies of HIV.
- *Transcription:* When the host cell is activated, the provirus integrated in the host genome is also transcribed by host’s RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins.
- *Assembly:* An HIV enzyme “protease” cuts the long chains of HIV proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of HIV's RNA genetic material, a new virus particle is assembled.
- *Budding:* The newly assembled virus pushes out (“buds”) from the host cell. During budding the HIV envelope also acquires host membrane proteins and lipid bilayer (Fig 1.3).

1.3 Pathogenesis of HIV

Natural history of HIV-1 infection encompasses an acute/ primary phase that lasts for months, followed by a clinically latent phase that typically lasts for few years and ultimately by the collapse of immune system that characterized AIDS. Pattern of commonly detected HIV-specific immune responses and plasma viral RNA level is shown in Fig. 1.4.

Figure 1.3: *HIV life cycle*

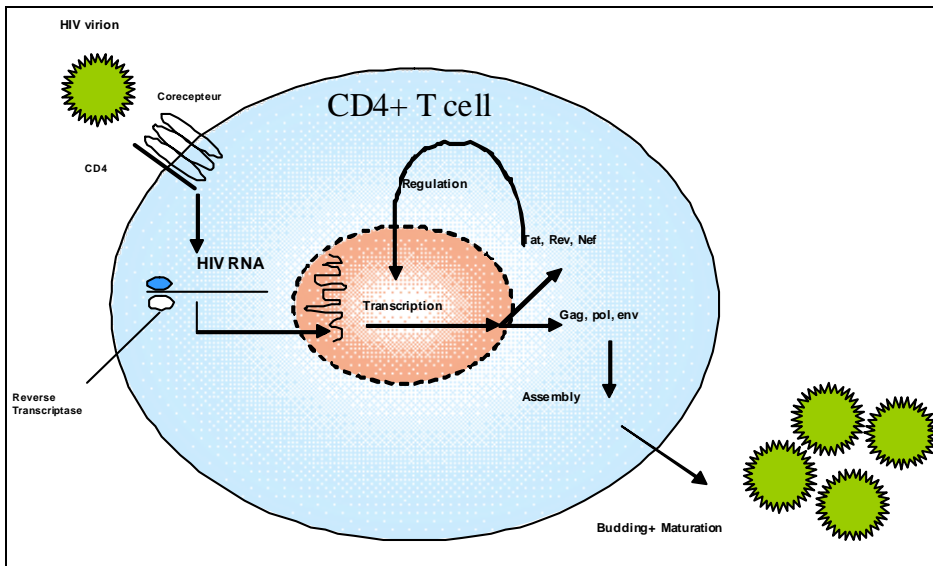
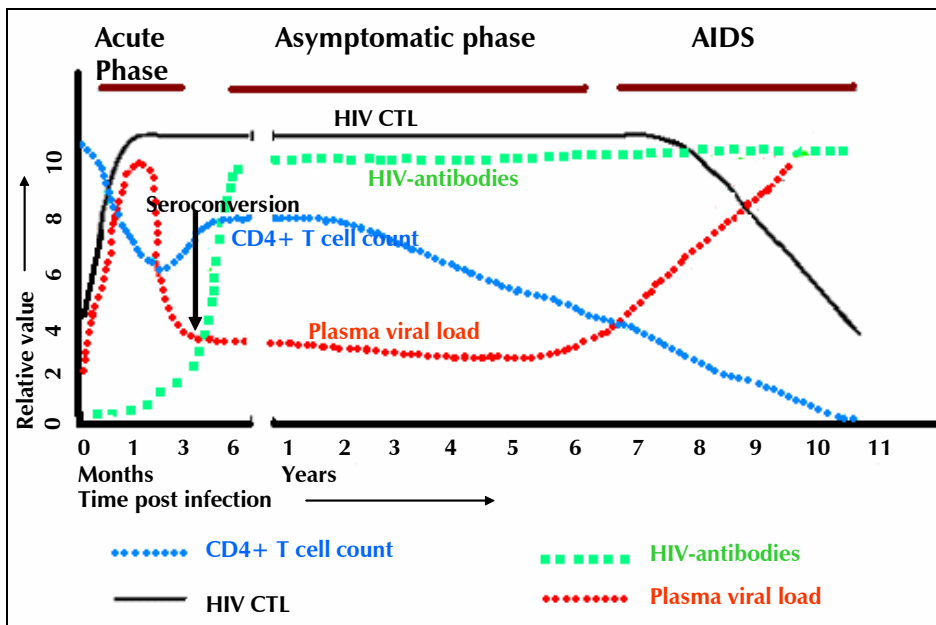


Figure 1.4: *Immunological and virological events in natural course of HIV infection*



Acute phase of HIV-1 infection (Window period)

From the time one gets infected with HIV-1, it may take three to six weeks for anti-HIV antibodies appearing in peripheral circulation. This period is called “window period”. The diagnostic tests that detect anti-HIV antibodies are negative during this period. However, this is a very important stage in HIV pathogenesis. There is surge of viraemia with plasma viral load reaching peak in 2-3 weeks and loss of T helper cells during this period causing a transient drop in circulating CD4 T lymphocytes. The host generates immune response during this period that helps in controlling the virus multiplication leading to sharp decline in plasma viraemia. A proportion of infected person may suffer a flu like clinical disease during this period. The patient may report with fever, headaches, arthralgia, rashes etc. However, acute primary HIV disease is usually self limiting.

About 4-6 months post-infection, steady state of viraemia (virologic set point) is achieved in each patient. The plasma viral load set point is prognostic for the future course of the disease. Low plasma virus load is usually associated with slower disease progression.

Post seroconversion asymptomatic (clinically latent) phase of HIV infection

The plasma virus load level remains stable for several years post plasma virus load set point. The infected person remains by and large asymptomatic during this period. Although plasma virus load levels do not rise, there is constant multiplication of virus leading to the destruction of CD4 T lymphocytes. The replenishment of CD4 T lymphocytes cannot keep pace with the loss of lymphocytes. As a result there is gradual drop of CD4 T lymphocytes in peripheral circulation. Plasma virus load and CD4 T lymphocytes counts are hence two important parameters of HIV disease progression.

The decline in CD4 T lymphocytes counts may be influenced by various factors such as plasma virus levels, opportunistic infections, nutritional factors etc. Based on the speed of disease progression, HIV-1 infected individuals can be categorized as typical progressors, rapid progressors and long term non-progressors. Typical progressors show latent period of 5 to 8 years before appearance of clinical AIDS. About 10% of

HIV-infected individuals rapidly progress to AIDS within 2 to 3 years after infection whereas less than 5% do not show clinical symptoms even after 10 years of infection in absence of anti-retroviral therapy. These are called long-term non-progressors.

AIDS stage

The gradual decrease in CD4 T lymphocytes cells (below 200 cells/mm³) ultimately results in loss of control over the immune response and various opportunistic infections start appearing. This is the terminal stage of HIV infection and called as AIDS. Common opportunistic infections include *Pneumocystis carinii*¹ pneumonia, cryptococcal meningitis, oral and oesophageal candidiasis. Recurrent activation of Herpes zoster and malignancies such as non-Hodgkin's lymphoma are more commonly seen during this phase. Symptoms such as night sweats, fever, diarrhoea, profound weight loss and fatigue are commonly seen during this period.

1.4 Factors influencing HIV disease progression

The rate of progression of HIV disease is usually influenced by a number of factors. Prominent among those are age at acquisition of HIV infection, genetic factors, other diseases such as tuberculosis and hepatitis, nutrition etc.

Genetic factors also influence the disease progression. One of the prominent example is mutation in CCR5 gene. Heterozygosity for $\Delta 32$ mutation in CCR5 gene is associated with slow disease progression. HLA alleles such as B27 and B57 are associated with slower progression of the disease. The influence of genetic factors on the disease progression has not yet been fully understood.

Besides genetic factors coinfections such as tuberculosis, Hepatitis B and helminthic infections also accelerate the disease progression. Malnutrition and vitamins and micronutrient deficiencies may also be associated with rapid progression to AIDS.

¹ This organism is now renamed as *Pneumocystis jiroveci* and classified as a fungus

1.5 WHO clinical staging of HIV-related disease in adults and adolescents aged 15 years or more

The revised WHO clinical classification of HIV-associated disease is designed to be used in patients with **confirmed HIV infection**. Along with measurement of the CD4 T lymphocytes count, where available, the staging system is used to guide decisions on when to start prophylaxis for opportunistic infections (OI) and when to start and switch antiretroviral therapy (ART).

Table 1.2: Revised WHO clinical staging of HIV-related disease in adults and adolescents aged 15 years or more

| |
|--|
| Clinical stage 1 (Asymptomatic) |
| Asymptomatic PGL |
| Clinical stage 2 (Mild disease) |
| Unexplained moderate weight loss (<10% of presumed or measured body weight) Recurrent respiratory tract infections (sinusitis, tonsillitis, otitis media and pharyngitis) Herpes zoster Angular cheilitis Recurrent oral ulcerations PPE Seborrhoeic dermatitis Fungal nail infections |
| Clinical stage 3 (Moderate disease) |
| Unexplained severe weight loss (>10% of presumed or measured body weight) Unexplained chronic diarrhoea for longer than one month Unexplained persistent fever (above 37.5 °C, intermittent or constant, for longer than one month) Persistent oral candidiasis Oral hairy leukoplakia (OHL) Pulmonary TB Severe bacterial infections (such as pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia) Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis Unexplained anaemia (<8 g/dl), neutropenia (<0.5 x 10 ⁹ /litre) or chronic thrombocytopenia (<50 X 10 ⁹ /litre ³) |

Clinical stage 4 (Severe disease)

HIV wasting syndrome
Pneumocystis jiroveci pneumonia (PCP)
Recurrent severe bacterial pneumonia
Chronic herpes simplex infection (orolabial, genital or anorectal, of more than one month's duration or visceral at any site)
Oesophageal candidiasis (or candidiasis of the trachea, bronchi or lungs)
Extrapulmonary TB (EPTB)
Kaposi sarcoma
Cytomegalovirus (CMV) infection (retinitis or infection of other organs)
Toxoplasmosis of the central nervous system (CNS)
HIV encephalopathy
Extrapulmonary cryptococcosis including meningitis
Disseminated non-tuberculous mycobacterial infection
Progressive multifocal leukoencephalopathy (PML)
Penicilliosis
Chronic cryptosporidiosis
Chronic isosporiasis
Disseminated mycosis (extrapulmonary histoplasmosis, coccidioidomycosis)
Recurrent septicaemia (including due to non-typhoidal *Salmonella*)
Lymphoma (cerebral or B-cell, non-Hodgkin)
Invasive cervical carcinoma
Atypical disseminated leishmaniasis
Symptomatic HIV-associated nephropathy or HIV-associated cardiomyopathy

PGL: persistent generalized lymphadenopathy; PPE: pruritic papular eruptions;
OHL: oral hairy leukoplakia; TB: tuberculosis; CMV: cytomegalovirus;
CNS: central nervous system; PML: progressive multifocal leukoencephalopathy

Chapter 2

Role of CD4 T lymphocytes in disease progression

The CD4 T lymphocytes; a subpopulation of the lymphocytes also known as T helper cells, are coordinators of the body's immune response, e.g., providing help to B cells in the production of antibody, as well as in augmenting cellular immune response to antigens.

The “CD” or cluster of differentiation is a protein expressed on the surface of the cells of the hematopoietic system. The expression of these proteins is used in lymphocyte nomenclature. Over 300 CD molecules have been reported so far. These proteins are often associated with the specific function of the cells. Cells with different functions express different CD molecules (for eg: CD3+ cells are total T lymphocytes, CD4+ cells are T helper cells, CD8+ cells are cytotoxic T lymphocytes and CD19+ are B lymphocytes).

CD4 T lymphocytes occupy the central position in regulating immune functions. CD4 T lymphocytes are the primary targets of HIV. The relentless destruction of CD4 T lymphocytes by HIV, either directly or indirectly, results in the loss of HIV-specific immune response, recall antibody response and, finally, non-specific immune response in the AIDS stage.

2.1 CD4 T lymphocytes and HIV

Within hours of exposure to HIV, CD4 T lymphocytes are found to be infected showing active viral replication. The infected CD4 cells release virions by budding through the cell membrane or by lysis of the infected cells. The released virus particles then infect uninfected CD4 T lymphocytes. CD4 T lymphocytes also serve as important reservoirs of HIV: a small proportion of these cells carry HIV provirus integrated in the host DNA without active virus multiplication.

During the primary HIV infection, the number of CD4 T lymphocytes in the bloodstream decreases by 20% to 40%. HIV brings about the lysis of HIV

infected cells as well as bystander uninfected cells using various mechanisms such as lysis of the cells infected with HIV. Billions of CD4 T lymphocytes may be destroyed every day, eventually overwhelming the immune system's regenerative capacity. In acute HIV-1 infection, in addition to the decline in CD4 T lymphocytes counts, qualitative impairments of CD4 T lymphocytes function are detected. The impairment of HIV-1-specific CD4 T lymphocytes function occurs very early in acute infection. Following acute primary HIV infection, one may remain free of HIV-related illnesses, often for years, despite ongoing replication of HIV in the lymphoid organs and relentless destruction of the immune system. However, during the period, the immune system remains sufficiently competent to provide immune surveillance and to prevent most infections. Although the decrease in the total number of T lymphocytes marks the decrease in immune competence, sometimes the quantitative loss of CD4 T lymphocytes may not be matched by the qualitative functions. A number of assays such as cytokine induction, antigen-induced proliferation, measurement of activation markers etc can assess the functions of lymphocytes. However, the total CD4 T lymphocytes number still remains the most robust marker of immune competence.

The progressive loss of CD4 T lymphocytes eventually results in the loss of an ability to mount desirable immune response to any pathogen and vulnerability to opportunistic pathogens characteristic of AIDS. The estimation of peripheral CD4 T lymphocytes counts is relied upon for taking a decision on initiation of ART.

The estimation of peripheral CD4 T lymphocytes counts has also been used as a tool for monitoring disease progression and the effectiveness of antiretroviral treatment(ART). The changes in the CD4 T lymphocytes counts are important indicators of the response to ART. HIV plasma virus load is a sensitive indicator of the progression of HIV disease. However, due to the relatively high cost of virus load estimation, the CD4 T lymphocytes count remains the most important key indicator for initiation and monitoring of ART and a measure of the effectiveness of the treatment in clinical trial evaluations.

2.2 CD4 T lymphocyte counts and antiretroviral therapy

CD4 count not available

In the absence of facilities for CD4 T lymphocytes count, all patients with WHO stages 3 and 4 disease should start ART. Those with WHO stages 1 and

2 disease should be monitored carefully, with a minimum of three-monthly clinical reviews and at any time if new symptoms develop (Table 2.1).

Table 2.1: Starting antiretroviral therapy based on clinical staging

| WHO clinical stage | Recommendation |
|--------------------|----------------|
| 1 | Do not treat |
| 2* | Do not treat |
| 3 | Treat |
| 4 | Treat |

*Consider starting treatment in patients with WHO stage 2 disease and TLC <1200 cells/mm³.

CD4 count available

The optimum time to commence ART is prior to patients becoming unwell or presenting with their first OI. Disease progression is greater in patients who commence ART with a CD4 T lymphocytes count <200 cells/mm³ compared with those who start therapy at counts above this level. If facilities for CD4 T lymphocytes count measurement are available, ART should be started before the CD4 count drops below 200 cells/mm³. The optimum time to initiate ART in patients with a CD4 cell count of 200–350 cells/mm³ remains unknown, and patients with CD4 counts in this range require regular clinical and immunological evaluation.

Initiation of ART is recommended for all patients with pulmonary TB or severe bacterial infections and CD4 counts <350 cells/mm³. Initiation of ART is also recommended for all pregnant women with any stage 3 disease and a CD4 count <350 cells/mm³ (Table 2.2).

The decision to initiate ART in adults and adolescents is based on clinical and immunological assessment. In many resource-limited settings, clinical staging alone will be available to guide the decision of when to start ART. Measuring viral load (HIV RNA) is not recommended to guide decision on when to start ART.

In the era of highly active anti-retroviral therapy, it has been possible to reverse the decline in CD4 T lymphocytes numbers as a result of potent anti-HIV treatment and control of plasma viremia. As untreated HIV

infection progresses, the CD4 T lymphocytes count declines about 4% per year. In response to successful ART, the CD4 T lymphocytes count typically increases by >50 cells/ μ L within weeks after viral suppression, and then increases 50-100 cells/ μ L per year thereafter until a threshold is reached. In some patients, CD4 T lymphocytes counts may not increase as quickly or as steadily, even with durable viral load suppression.

Table 2.2: Starting antiretroviral therapy by CD4T lymphocytes count

| WHO clinical staging | CD4 count available |
|----------------------|---|
| | 1 |
| 2 | |
| 3 | <p>General principles</p> <ul style="list-style-type: none"> • Consider treatment if CD4 count <350 cells/mm³ but initiate before CD4 count drops below 200 cells/mm³ <p>In the case of pregnancy or TB</p> <ul style="list-style-type: none"> • Start ART in all HIV-infected pregnant women with WHO stage 3 disease and CD4 count <350 cells/mm³ • Start ART in all HIV-infected patients with CD4 count <350 cells/mm³ and pulmonary TB (WHO stage 3) or severe bacterial disease |
| 4 | Treat irrespective of CD4 count (extrapulmonary TB is WHO stage 4 disease) |

2.3 CD4 T lymphocytes counts as indicator of treatment failure

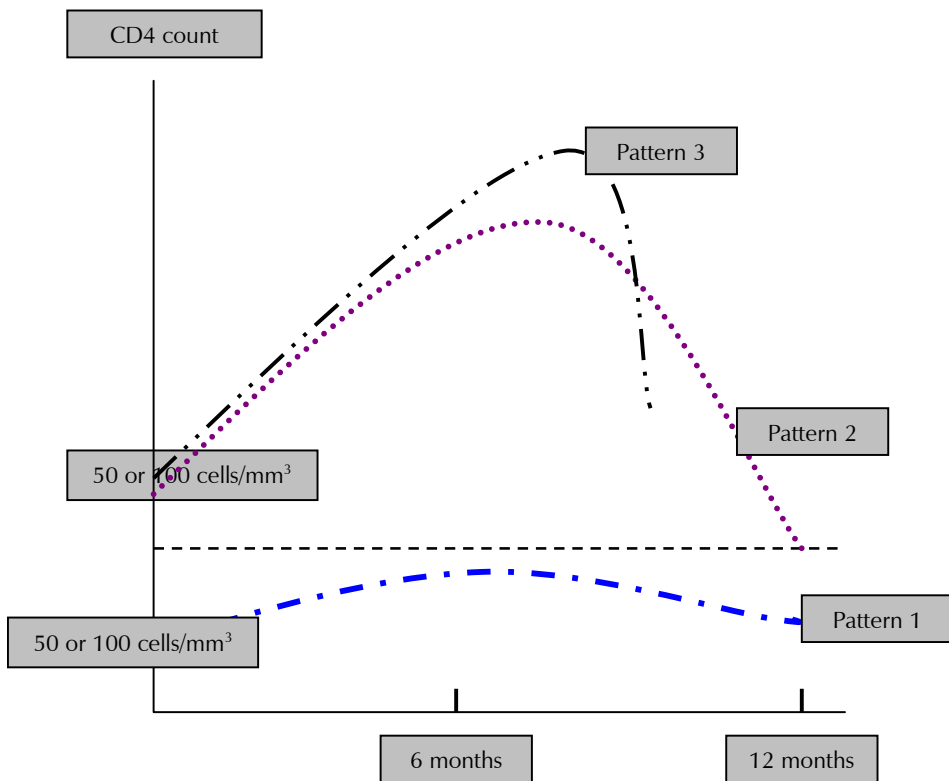
Treatment failure can be ascertained on the basis of clinical, virological and immunological evaluations.

Clinical failure: New or recurrent WHO stage 4 condition after at least 6 months of ART. Exceptions are TB, oesophageal candidiasis and severe bacterial infections which may not always represent ART failure.

Virological failure: Viral load >10 000 copies/ml after at least 6 months on ART.

Immunological criteria for failure: These have been shown in Fig 2.1

Figure 2.1: **Immunological criteria for failure**



Pattern 1: CD4 count <100 cells/mm³ (some experts recommend <50 cells/mm³) after one year of therapy

Pattern 2: Return to or a fall below the pre-therapy baseline CD4 count after one year of therapy

Pattern 3: 50% decline from the on-treatment peak CD4 value (if known)

CD4 cell count can also be used to determine when not to switch therapy. For example, in a patient with a new clinical stage 3 event for whom a switch is being considered, switching is not recommended if the CD4 cell count is >200 cells/mm³.

Virological criteria for failure are included here as some countries in the Region (such as India and Thailand) have increasing capacity to perform affordable viral load testing. Viral load remains the most sensitive indicator of ART failure. Recognizing early failure facilitates switching before multiple resistance mutations have developed to drugs in the first-line regimen. The optimal viral load value at which ART should be switched has not been defined. However, values >5000 – $10,000$ copies/ml have been associated with subsequent clinical progression and appreciable CD4 cell count decline.

2.4 CD4 T lymphocytes counts in paediatric population

The rate of disease progression amongst untreated HIV-infected infants is much higher than in adults.

During early paediatric age, the total lymphocyte counts and the absolute CD4 T lymphocytes counts are higher than seen in the adult population. There is a gradual decrease in counts till they reach the adult normal range. WHO classification for CD4 T lymphocytes in HIV-associated disease in children is shown in Table 2.3.

Table 2.3: WHO classification of HIV-associated immunodeficiency using CD4 count

| Classification of HIV-associated immunodeficiency | Age-related CD4 values | | | |
|---|------------------------|---------------------|---------------------|---|
| | ≤ 11 months (CD4%) | 12-35 months (CD4%) | 36-59 months (CD4%) | ≥ 5 years (cells/mm ³ Or CD4%) |
| Not significant | > 35 | > 30 | > 25 | > 500 |
| Mild | 30-35 | 25-30 | 20-25 | 350-499 |
| Advanced | 25-29 | 20-24 | 15-19 | 200-349 |
| Severe | < 25 | < 20 | < 15 | < 200 or < 15% |

Younger children normally have higher CD4 counts than older children and adults. CD4% is the preferred measurement in children <5 years old, as it varies less in them than in older children. At ≥ 5 years of age, either CD4% or absolute CD4 count can be used but CD4 count is preferred.

The total leucocyte count (TLC) is an option that is used only if CD4 measurement is not available in children with WHO clinical stage 2 disease (Table 2.4). It cannot be used in asymptomatic children. The TLC is also not useful for monitoring ART.

It is also important to have the established reference ranges of CD4 T lymphocytes and other lymphocyte subsets. However, no normal ranges have been established in children in South-East Asian countries.

Table 2.4: Diagnosing severe immunodeficiency in children using TLC

| Classification of HIV-associated immunodeficiency | Age-related CD4 values | | | |
|---|------------------------|--------------|--------------|-----------|
| | < 11 months | 12-35 months | 36-59 months | ≥ 5 years |
| TLC | < 4000 | < 3000 | < 2500 | < 2000 |
| CD4 count | < 1500 | < 750 | < 350 | < 200 |

Chapter 3

Principles of flow cytometry

3.1 Introduction

The advent of monoclonal antibody technology and the development of flow cytometry have facilitated the phenotypic characterization of functionally different types of lymphocytes. In addition, the availability of multi-parametric flow cytometry using a variety of fluorochrome has made it possible for simultaneous measurements of large numbers of subsets of cells. Flow cytometry has added a new dimension to medical and biological research, including the immunology of HIV. This chapter describes the working of this versatile technology.

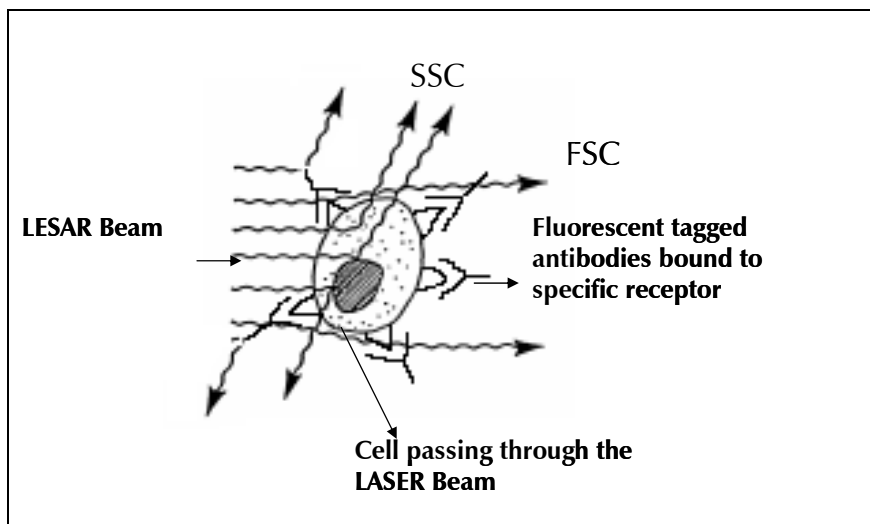
3.2 What is flow cytometry?

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.

Individual cells stained with fluorescent labels or absorption dyes are suspended in physiological solution and introduced under a slight pressure through a flow chamber into the centre of a stream of cell-free sheath fluid. The light scattered by the individual particle and the fluorescence emitted by the cells is used for analysis and sorting of the cells based on the fluorescent antibody directed against a specific surface. This combination of scattered and fluorescence light is picked up by the detectors in the flow cytometer. These detectors then produce electronic signals that are proportional to the optical signals received.

The visible light undergoes deflection based on the size and internal structures of the cell. *FSC (Forward Scatter)* correlates with the cell volume. *SSC (Side Scatter)* depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). The fluorescence emitted by the cell depends upon the fluorescence tagged specific monoclonal antibodies against the cell surface markers (Fig 3. 1). The data collected on each cell or event are stored in the computer. This data is then processed and analyzed to provide information about cell populations within the sample.

Figure 3.1: **Generation of Scatter (FSC & SSC) and Fluorescence**

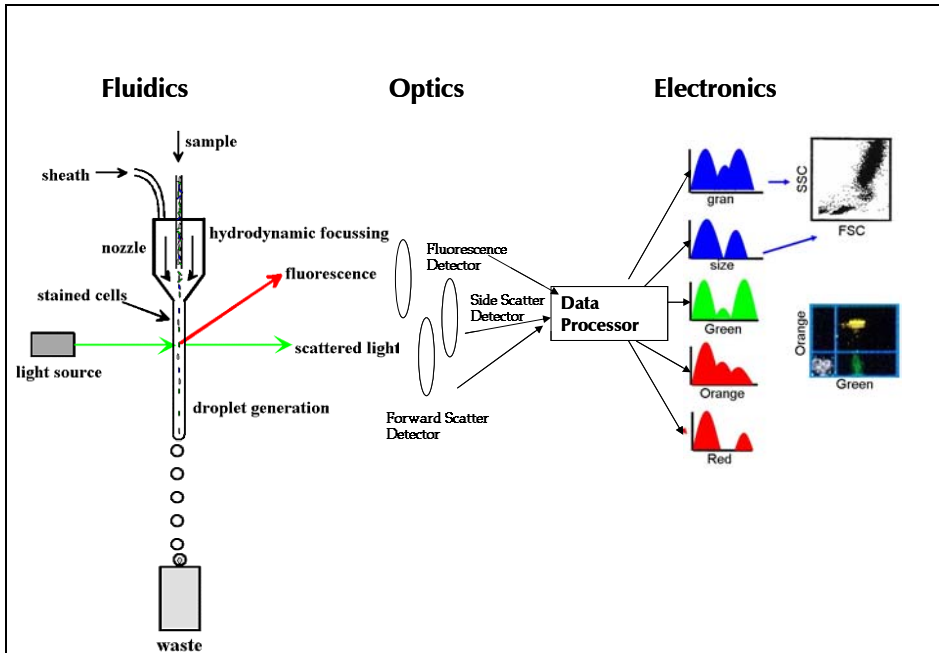


3.3 Instrument description

A flow cytometer is generally made up of three main systems (Fig 3.2):

- fluidics,
- optics, and
- electronics.

Figure 3.2: **Simplified schematics of flow cytometry**



Fluidics system

The purpose of the fluidics system is to transport cells in a fluid stream through the laser beam for interrogation. Any cell ranging from 0.2 to 100 μm in size is suitable for flow cytometer analysis. 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. The flow of sheath fluid accelerates the cells and restricts them to the centre of the sample core where the laser beam will then interact with the cells. The cells are ejected through this flow chamber with a velocity of about 10 m/sec or at a rate of some 1000 cells/sec.

For qualitative measurements such as immunophenotyping of positive fluorescent cells and negative fluorescent cells, a higher sample pressure or flow rate is generally used. Whereas, in applications where greater optical resolution is critical, such as DNA analysis a lower rate is generally used.

Optics system

The optics system is a complex system of lenses which consists of two major parts: 1) excitation or illumination optics, 2) collection optics. The excitation optics in flow cytometer is light sources and lens. Light sources can be either lasers (e.g. an argon ion laser with 488 nm single line emission), or conventional lamps (e.g. mercury arc lamp). Light output from laser is monochromatic, unidirectional and in phase. Lasers are used to excite or illuminate the cells in the sample stream, whereas the lens are used to shape and focus the laser beam. Once the cells of interest intercept the light source, they scatter light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes (e.g. fluorescein isothiocyanate (FITC) excited by 488 nm argon ion laser light emits 530 nm of green fluorescence).

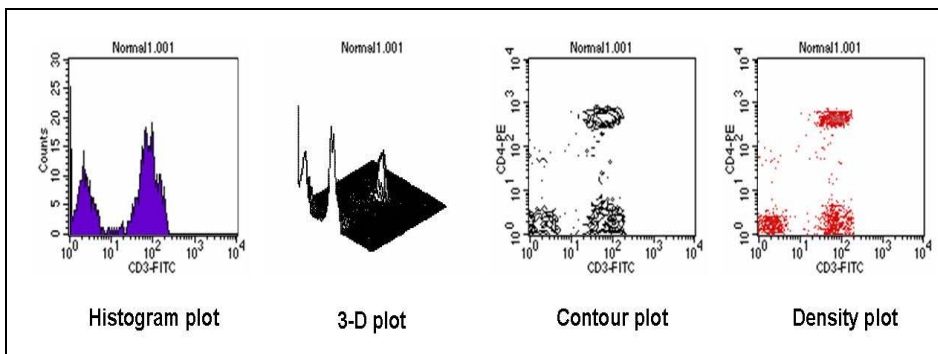
The collection optics or photodetectors consists of a collection lens to collect light signals emitted from the cells. There are forward scatter (FSC) collection optics, fluorescence and side scatter (SSC) collection optics. In general, flow cytometer has three types of photo detectors: 1) a FSC detector is a relatively sensitive 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. 2) The SSC detector receives refracted and reflected light signals, which are proportional to cell granularity or complexity. This SSC is detected at 90° to incident light axis. 3) Fluorescent light is collected by a lens and divided between two photomultiplier tubes (PMT) detectors either by a semi-silver mirror or a dichroic beam splitter. Placing an optical filter in front of the PMT optimizes the specificity of a detector for a particular fluorescence. Each optical barrier filter allows only a narrow range of wavelengths to reach the detector.

Electronics system

The electrical pulses originating from light detected by the PMTs are then processed by a data processor using a series of linear and log amplifiers. 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 in cells. This type of amplification expands the scale for weak signals and compresses the scale for “strong” or specific fluorescence signals.

After the different signals or pulses are amplified they are processed by an Analog to Digital Converter (ADC), which, in turn, allows for events to be plotted on a graphical scale (Fig 3.3). Data corresponding to one sample can be stored as a histogram file and/or list-mode file. The data storage file includes a description of the sample acquired, the instrument on which the data was collected, the data set, and the results of data analysis.

Figure 3.3: **Flow cytometry data can be displayed as histogram, 3-dimension, and contour plots as well as density plot**



3.4 Use of fluorochromes in flow cytometry

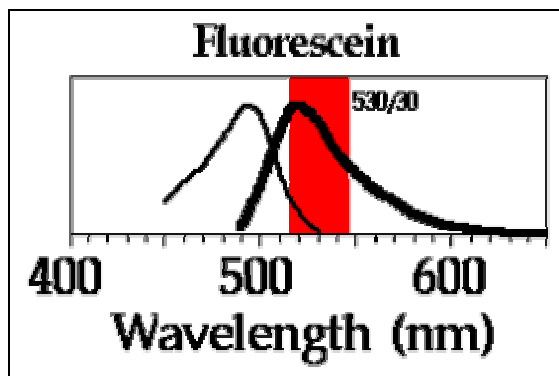
A fluorochrome is a chemical compound that absorbs light energy over a range of wavelength that is characteristic for that fluorochrome. Once the fluorochrome absorbs light, it causes an electron in the fluorochrome to go to a higher energy state. This excited electron or energy quickly decays to its ground state, and in the process the excess energy is released as a photon of light with unique spectral property. This transition of energy is called fluorescence. The range of emitted wavelengths for a particular fluorochrome is called its emission spectrum. There are several sources of laser with different excitation wavelengths such as, Argon ion laser, Krypton ion laser, Helium-Neon laser and Helium-Cadmium as well as Mercury arc lamp. The Argon ion laser is commonly used in flow cytometer because its emission excites more than one fluorochrome.

The two most commonly used fluorochromes are FITC and phycoerythrin (PE). These are easily attached to antibodies or proteins by reaction of its isothiocyanate derivatives with protein amino groups. They are

ideally suited to excitation by the argon-ion laser line at 488 nm. FITC on excitation emits green fluorescence. PE, among a group of phycobiliproteins, can also be used to label antibodies for studying cell surface antigens. Its strong visible absorption band excites efficiently over a fairly wide range of wavelength from 440 nm to 580 nm with its emission maximum at 575 nm. PE, on excitation, emits orange fluorescence. Both FITC and PE fluorochrome are used in combination since the peak emission wavelengths of these two fluorochromes are far enough apart so that each signal can be detected by a separate photodetector. The amount of fluorescent light detected is proportional to the number of fluorochrome molecules on the cells.

Several fluorochromes show spectral overlap of fluorescent emissions (Fig 3.4). This leads to incomplete optical separation by filters. This fluorescent overlapping often happens when the same wavelength of excitation is used. An example is the popular combination of FITC and PE excited by 488 nm Argon ion laser light. Fluorescence emission from FITC has its peak emission at 525 but has around 20%-30% contaminated in the emission area of PE. The wavelength of maximum emission of PE fluorescence is around 575 and has about 1%-2% of emission contamination in the emission area of FITC. To compensate this crosstalk, a fraction of FITC sensor output signal is subtracted from the output of the PE-output signal and vice versa. This electronic subtraction is called compensation.

Figure 3.4: *Overlap in fluorescent emission*



Chapter 4

Methods of CD4 T lymphocytes count measurement

4.1 Introduction

Several technologies for determining the absolute number of CD4 T lymphocytes have been developed and evaluated in multicentric studies. The technologies are either flow cytometric or non-flow cytometric. The choice of the methods depends upon multiple factors.

4.2 Flow cytometric methodologies

Immunofluorescence analysis by flow cytometry is the gold standard for CD4 T lymphocytes measurements and also the method of choice if a large throughput of samples is required. The principles of flow cytometry have been already discussed in Chapter 3 in detail. The flow cytometric assays work on the principle of scattering of light due to different sizes, granularity of the cells passing through the laser beam, and also by the fluorescence emitted by the cells after staining with the specific monoclonal antibodies to cell surface markers that are tagged with different fluorescence dyes. The population of interest can be thus identified and gated (Fig.4.1) for further analysis within the population of interest. The monoclonal antibodies specifically bind different surface receptors like CD4 for T helper cells. Relative percentages of the cells expressing the specific receptor (eg. CD4) on its surface are obtained from the flow cytometer and the absolute counts can be calculated with the help of absolute lymphocyte count obtained from haematology analyzer as detailed below.

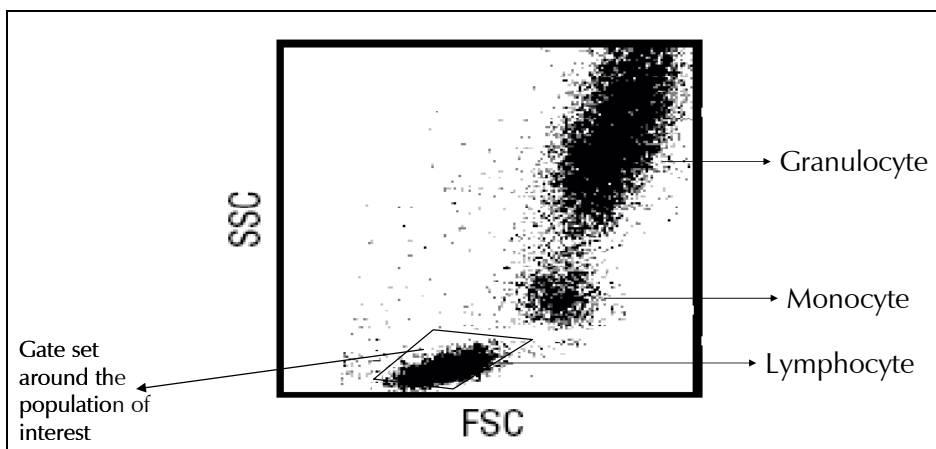
Absolute CD4 T lymphocytes count determination by flow cytometer may be performed using dual- and single-platform methods.

Dual-platform approach

The dual-platform approach estimates absolute CD4 T lymphocytes counts by a mathematical formula using two independent parameters; CD4 T

lymphocytes percentage obtained by flow cytometer and total (WBC) and differential lymphocyte counts estimated by a haematology analyzer or a haemocytometer. The lymphocyte population can be gated (identified) in a flow cytometer using the suitable surface markers for leucocyte subpopulations (Fig 4.1) and the percentage of CD4 T lymphocytes amongst the total lymphocyte percentages i.e. gated population can be obtained.

Figure 4.1: **Lymphocyte gating using forward scatter and side scatter parameters**



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 use this strategies are described elsewhere.

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.

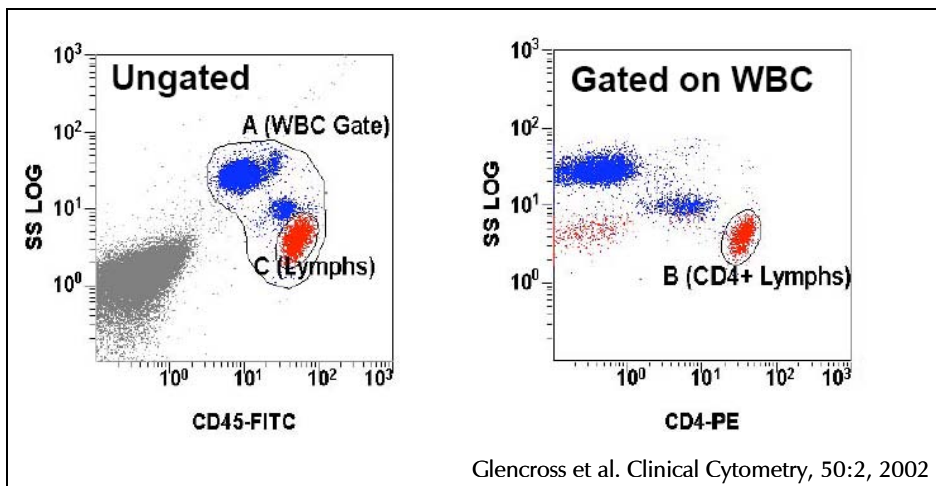
Panleucogating

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. 4.2)

Figure 4.2: **Flow cytometric display of the panleucogating approach for CD4 count estimation**



Problems encountered with dual platform methodologies

The dual-platform approach requires use of two different equipments for obtaining absolute CD4 counts. The use of a haematological analyzer as the second platform for white blood count and the three- or five-part differential count introduce variable factors into the calculation of absolute CD4 T lymphocytes. In the individual patient, variation in the total white blood counts and lymphocyte differential over time leads to the greater variability of the absolute CD4 T lymphocyte counts compared with %CD4+ T-cells. Moreover, the patient samples contain cell numbers beyond the sensitivity

and linearity response range of haematological analyzer (e.g. in severely leucopenic patients), the lymphocyte population defined by flow cytometer may not match exactly with that of the hematological analyzer. Nevertheless, this dual-platform technology is still widely practiced and recommended in a number of institutional guidelines.

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.

Single-platform approach

The single-platform approach enables absolute CD4 T lymphocytes counts to be derived directly without the need for a haematological analyzer. This can be assessed either by counting CD4 T lymphocytes populations in a precisely determined blood volume or by using the known numbers of fluorescent microbeads admixed to a known volume of CD4-stained blood. It is required to pipette small amounts of reagents, e.g.: 10 μ l to 25 μ l and hence the pipetting technique is very crucial for reliable use of the single-platform approach.

To date, many single-platform technologies have been developed commercially. The details of both microbead-based technologies and the volumetric technologies are given below.

A. FACSCount microbead-based system

FACSCount system is the product of Becton Dickinson Biosciences (Fig. 4.3). It is the only available microbead-based single-platform instrument that is designed specifically for enumerating the absolute CD4+, CD8+ and CD3+ T-cell counts in no-lyse, no-wash whole blood. This system requires ready-to-use, twin-tube reagent tubes: One tube determines the absolute number of helper/inducer T-cells (CD4/CD3) or CD4+ T-cells by using a combination of two-colour monoclonal anti-human CD3 antibody conjugated to the tandem dye phycoerythrin +Cy5 (PECy5) and a monoclonal anti-human CD4 antibody conjugated to phycoerythrin (PE). The other tube determines the absolute number of Cytotoxic/suppressor T-cells (CD8-PECy5/CD3-PE) or CD8+ T-cells. Both tubes also give the absolute number of total T-cells (CD3) or CD3+ T cells, as well as CD4/CD8 ratio. In addition to the antibody reagents, the reagent tubes also

contain a known number of fluorochrome-labeled reference beads. These beads function as fluorescence standard for locating the lymphocytes and also as a quantitation standard for enumerating the cells. The control set consists of fluorescent beads at four different densities: zero (0 beads/ μL), low (50 beads/ μL), medium (250 beads/ μL), and high (1000 beads/ μL).

When whole blood is added to the reagents, fluorochrome-labeled antibodies in the reagents bind specifically to lymphocyte surface antigens. After a fixative solution is added, the sample is run on the instrument. Here the stained cells come in contact with the green HeNe laser light, which causes the cell to fluoresce. This fluorescent light provides the information necessary for the instrument to count the cells. The calculation of absolute CD3+, CD4+ and CD8+ T-cells is determined automatically by using the built-in Attractors software programme.

The system is robust and requires minimal training and hence can be used in a laboratory where the sample load is <50 samples /day. The system can provide absolute CD4 counts and for obtaining the percentage of CD4 cells, the ALC from the Haematology analyzer has to be used.

*Figure 4.3: FACSCount microbead-based system
No-lyse, no-wash procedure*



B. Modified flow cytometry:

The CD45 gating approach combined with the use of fluorescence beads in the procedure has converted the standard flow cytometry into a single platform approach. This single-platform system eliminates the need for multiple technologies (i.e., flow cytometry and haematology) and should be less expensive than the available methods when labour, cost and inconvenience of repeat samples, and haematology costs are considered. These absolute-count tubes contain a lyophilized pellet that dissolves during sample preparation, releasing a known number of fluorescent beads. By gating the bead population during analysis, absolute cell counts can be readily determined by a simple calculation. Both, TruCount tubes (BD Biosciences) and Flow-Count fluorospheres (Beckman Coulter) provide lymphocyte subset percentages as well as absolute counts. The determination of both of these clinical parameters is important because the CD4 lymphocyte percentage is often required for monitoring paediatric HIV-positive populations.

C. Guava EasyCD4 volumetric system

The Guava EasyCD4 system (Guava Technologies) is a desktop modified flow cytometry based on a microcapillary cytometry technology (Fig 4.4). The flow cytometer is coupled with laptop computer for analysis. The system uses a diode green laser as a light source. The reagent kit consists of two monoclonal antibody directly conjugated to PECy5 and PE for CD3 and CD4 T-cell antigens. The procedure does not require sheath fluid and the system's sampling precision depends on the integrity of the fluid pathway. The system has been evaluated by CDC and others as part of a five-site validation study and also as a part of a four-site validation study for US-FDA submission. The US-FDA has approved the system.

D. Partec CyFlow counter (volumetric system)

CyFlow is another desktop single-platform technology made by Partec, Germany (Fig 4.5). It is a volumetric software controlled absolute count system equipped with either a single 532 nm green solid-state laser used for one fluorescence parameter or two lasers with a mercury arc lamp applicable for 2 or 3-color analyses. 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. It works on simple no lyse and no wash protocol. However, the robustness and reproducibility is under evaluation.

Figure 4.4: *Guava EasyCD4 volumetric system*
Lyse no-wash procedure

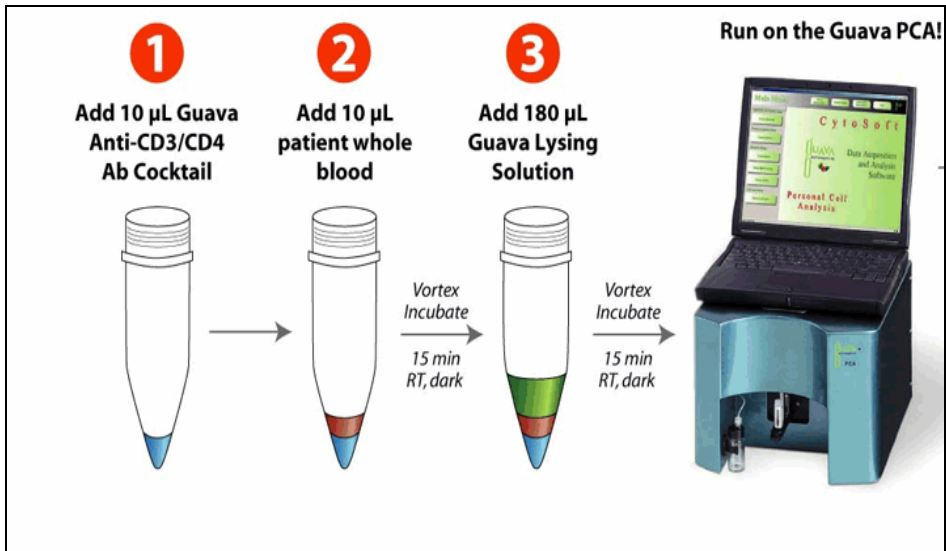
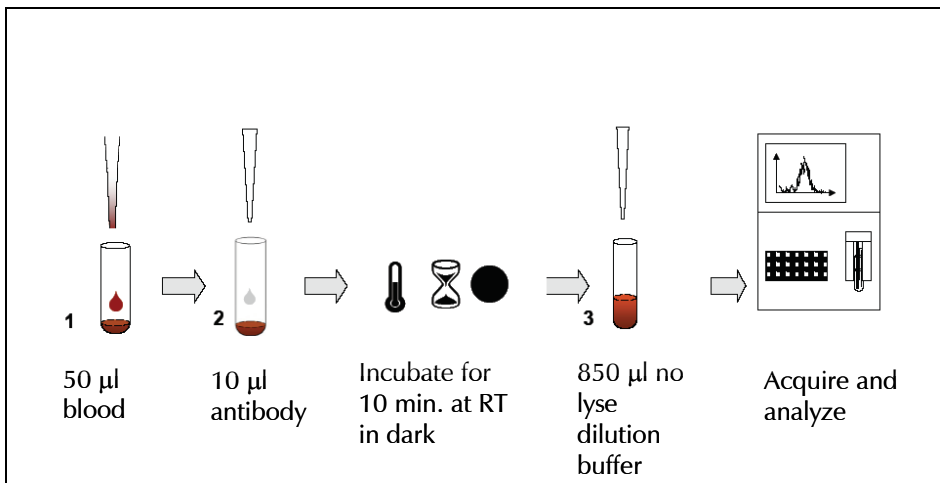


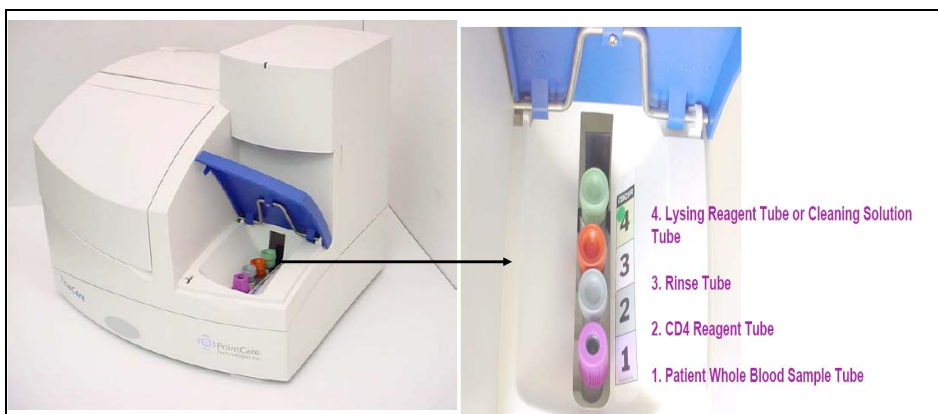
Figure 4.5: *Partec CyFlow count (volumetric system)*
No-Lyse, no-wash procedure



E. PointCARE system

This new flow Care system has been developed by Pointcare Technologies (Fig 4.6). It uses light scattering parameters and not the fluorescence dyes to distinguish lymphocytes from white blood cells. The CD4 T lymphocytes labeled with a colloidal gold-coupled antibody are then identified on the basis of wide-angle scatter. The entire system has been automated right from blood collection. A whole blood sample of 3-4 ml is placed in the machine and after a single button operation CD4 results are reported in approximately 17 minutes. The instrument provides absolute CD4 T lymphocytes counts, CD4 percentage, total leucocyte count, total lymphocyte count and lymphocyte parentage. The system has been approved by US-FDA recently. It is a mobile machine and can work on battery back-up. However the system is a closed system and the samples have to be processed in a very short time (4 hours of collection).

Figure 4.6: **PointCARE system**



4.3 Selections of methodology for CD4 T lymphocytes count estimation

For efficient and optimum reporting of CD4 counts the proper selection of the methodology is essential (Table 4.1). The choice of the assay should depend on:

- 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)
- Sample turnover (no. of samples 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 and equipment (regular maintenance is necessary)
- COST: The cost should include instrument and reagent cost as well as hidden cost of labour (often less expensive), disposables (if available often more expensive), shipping costs, infrastructure, repeat assay run and instrument repair
- The time within which the assay can be performed from the time of blood collection.

It is always beneficial to negotiate the price at an international or country level with bulk procurement schemes. It is also necessary to participate in a quality control programme to impart reliability to the results. While technologies are tested extensively by the manufacturers, additional validation process using established methods and instrumentation is highly recommended. Hence, technologies should be carefully reviewed and the needs of the lab considered in the purchase decisions. Technologies that have not been adequately validated should not be purchased.

4.4 Importance of reference ranges in interpretation of the CD4 T lymphocytes counts obtained by in the laboratory

Information on variability in the CD4+ T cell count in absence of HIV infection is very important in interpreting the result of CD4+ T cell count enumeration. The reference values hence are necessary for the given population as the values might vary from population to population depending upon the age, sex and race of a population, and even the

instruments the laboratory uses to perform the tests. The range is usually defined as the set of values 95% of the normal population falls within and commonly referred to as the *normal range* or *normal values*. However it has to be kept in mind that by *definition* 5% of the *normal* population will fall outside the reference range.

Table 4.1: Summary of flow based assay for CD4+ T cell count enumeration

| System | Parameters measured | Advantages | Disadvantages | Cost of the equipment US\$* | Cost/unit test* |
|--|---|--|---|-----------------------------|-----------------|
| Standard flowcytometry (method of choice) with different gating strategies: | | | | | |
| Standard flowcytometry (method of choice) | <ul style="list-style-type: none"> Absolute Count and percentages of CD4 CD3 and CD8 + T cells and other lymphocyte subsets for eg T, B and NK cells | <ul style="list-style-type: none"> Percentages from the flow cytometer Absolute counts using as a dual platform system with hematology analyzer. Different gating strategies can be used for gating the population of interest Can be used as a single platform system when the bead containing tubes are used to obtain the absolute count from the flow cytometer. Can be automated High Through put system Open system (can be used with reagents form various sources) | <ul style="list-style-type: none"> High cost of instruments and reagent systems Technically demanding Requires controlled utilities and environmental conditions Need good technical support Requires hematology analyzer for dual platform approach | 40000-80000 | 8-20 |
| Gating on the basis of size and granularity (Light scatter gating) | Same as above | Gating can be universal since no fluorescence marker is used for gating | Can not be used on aged samples Purity for the gated population is questionable | | |
| T cell (CD3) gating (lineage gating) | <ul style="list-style-type: none"> CD4, CD8 and CD3 percentages and absolute count | <ul style="list-style-type: none"> Accurate gating of the population of interest | <ul style="list-style-type: none"> Can not be used on aged samples | | |

| System | Parameters measured | Advantages | Disadvantages | Cost of the equipment US\$* | Cost/unit test* |
|--|--|---|---|-----------------------------|-----------------|
| Panleucogating approach (CD45/CD4 antibodies) | <ul style="list-style-type: none"> CD4+ T cell and Lymphocyte percentage Absolute CD4+ T cell count and lymphocyte with trucount (BD) or flowcount (Coulter) tubes | <ul style="list-style-type: none"> Single tube approach can be used accurately on the aged samples (upto 5 days) High throughput (upto 300-400 samples per day on a single instrument – 8 hour/day) Most widely used approach Can be performed with no lysis (lysis of RBCs) protocol | | – | US \$ 2-3 |
| Single platform approach | | | | | |
| FACSCount With microbead-based system (Becton Dickinson) | <ul style="list-style-type: none"> CD4, CD8 and CD3 + T cell absolute count CD 4/CD8 ratio | <ul style="list-style-type: none"> Fully automated No RBC lysis required Low-level expertise required US FDA approved Accuracy and precision validated Can be used on blood up to 48 hours after collection 100 uL whole blood/test | <ul style="list-style-type: none"> No CD4 percentage capability, important for monitoring infants and children Dedicated and closed system Process time (1 hour 30 minutes) High cost | US \$ 20000 | 5-20 |
| Guava EasyCD4 volumetric system | CD4, CD8 and CD3+ T cell absolute count | <ul style="list-style-type: none"> Robust Open system US FDA approved | <ul style="list-style-type: none"> Not validated adequately Information on the performance on stabilized samples is not available | 35000 | 5 |
| Partec Cyflow counter Volumetric system | <ul style="list-style-type: none"> CD4, CD8 and CD3+ T cell absolute Count CD4: CD8 ratio Other subsets depending upon reagents | <ul style="list-style-type: none"> No lyse system for absolute counts Volumetric cell counting, no bead calibrators required Open software architecture for menu expansion | <ul style="list-style-type: none"> No CD4 percentage capability if no-lyse method is used No independent validation of performance | 22000 | 8-10 |

| System | Parameters measured | Advantages | Disadvantages | Cost of the equipment US\$* | Cost/unit test* |
|------------------|---|---|--|-----------------------------|------------------------|
| | | <ul style="list-style-type: none"> • 15 minutes turn around time • lyse no-wash system can give percentage • Open system | <ul style="list-style-type: none"> • No model distinction for various CyFlow models • still under validation performance with stabilized samples is questionable | | |
| PointCARE system | <ul style="list-style-type: none"> • Absolute CD4+ T cell absolute count and percentage, Lymph% and absolute count | <ul style="list-style-type: none"> • No technical Skill required • Robust • Useful in remote places • US FDA approved | <ul style="list-style-type: none"> • Closed system, • Not validated adequately • Information on the performance on stabilized samples is not available | Still to be calculated | Still to be calculated |

*: The cost is subject to change with time

Chapter 5

Alternate methods for CD4 T lymphocytes estimation

5.1 Introduction

In developed countries, CD4 counts are typically performed every three to six months for each patient using the method of flow cytometry. Several factors—including the cost of a flow cytometer (which ranges from \$30,000 to \$150,000), technical and operational complexity, the need for reliable electric supply, and the high cost of reagents—have made these instruments impractical and/or difficult to sustain in resource-scarce settings. An ideal CD4 T lymphocytes testing in a resource-poor setting would be an assay which is simple, uses inexpensive instruments and requires a minimum training period that would reliably CD4 T lymphocytes without sacrificing much accuracy and precision. The urgent need for affordable and technically simple CD4 diagnostics is widely recognized and many such methodologies are available. This chapter gives an account of the available methodologies which are summarized in Table 1.

5.2 Manual methods

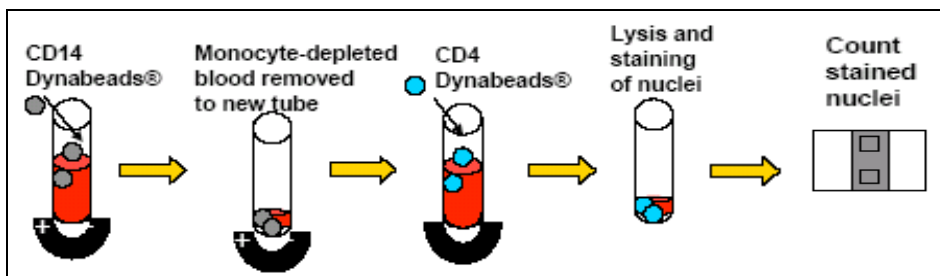
Microscope based

Low-cost, microbead separation of CD4 T lymphocytes from other blood cells, followed by standard manual cell counting techniques using a light microscope, offers lower reagent costs than standard flow cytometry. However, these methods are more subjective than the flow-based methods and there is no quality control programme available. These methods are still not approved by the US, FDA. These methods are low throughput and extremely labour intensive, and appear to be less accurate than traditional flow cytometry; thus, they have not been widely adopted.

Dynabeads CD4 T lymphocytes quantitation

This non-flow cytometric technology is the product of Dynal. The system uses the immunomagnetic cell isolation method which uses Dynabeads magnetic particles coated with antibody to CD4 and CD8 antigens to capture and isolate CD4 T lymphocytes and CD8 T lymphocytes from whole blood. The system uses two types of beads. The first bead (coated with CD14 antibodies) removes monocytes from the sample and the second bead (coated with CD4 or CD8 antibodies) estimates CD4 cells that get stained with acridine orange to make the cell nuclei visible for counting under a fluorescent microscope or on an automated analyzer (Fig 5.1). A modified DynaBeads system using an alternate stain for the cells can be used with a light microscope.

Figure 5.1: *Dynal assay using Dynabeads*



The system could be cheaper than other alternatives and will be useful in small settings if it is backed up with flow cytometry for quality assurance.

5.3 Methodologies likely to become available in the near future

Microchip-based systems

A new methodology using a microchip-based detection system, called an electronic taste chip (ETC) has been recently developed. The ETC can detect chemicals and proteins in solution. Each chip contains microspheres in a small chamber through which whole blood is passed. The microspheres are coated with monoclonal antibodies that attach to the surface proteins of lymphocytes such as CD4 T lymphocytes as they pass through the chamber. The chip array rests atop a fluorescent microscope connected to a charge-

coupled device (CCD). The CD4 T lymphocytes tagged with microspheres can be distinguished via this CCD camera and counted by computer software. Absolute counts can be obtained using this system. The system can be very useful in remote areas from where the images can be sent to a central lab. for viewing and comments.

Semi-Bio manual slide technology

This new system uses a slide with an anti-CD4 coated chamber that traps CD4 T lymphocytes during incubation and counts the cells after staining. The system is still under development.

These new technologies do not require technical skills and can be used in remote areas once validated on a large number of samples and having cleared the external quality assurance testing.

5.4 Non-CD4 markers for monitoring HIV disease progression and for initiation and monitoring the success of anti-retroviral therapy

Total lymphocyte counts

Total lymphocyte count (TLC) has shown a good correlation with CD4 T lymphocytes counts. TLC can be easily performed by automated haematology cell counter. In a light-scattering haematology cell counter, cells flow through a light beam rather than through an orifice. Different cell types intercepted by light will show different patterns based on the size and shape of the cells. In this way, the device can count the number of cells per second of flowing cells through the orifice or light beam, and because the volume flow rate can be measured one can determine the number of white blood cells per μl and or total lymphocyte count per μl of blood sample. It is an inexpensive methodology available at district level also. However, it has high variation due to the effect of other infections like helminthes, malaria etc. The EDTA interferes with the counts in the samples after 24 hours of collection. Various studies have shown 70% to 80% sensitivity of TLC to identify patients with CD4 counts $<350/ <200$ cells/cumm.

Table 5.1: Summary of alternate technologies for CD4 enumeration

| System | Parameters measured | Advantages | Disadvantages | Cost of the equipment US\$* | Cost/unit test* |
|--------------------------------|---------------------|---|---|-----------------------------|------------------------|
| Beckman Coulter Cytosphere kit | CD4 absolute count | Simple: Only microscope and counting chamber (0.1 mm deep) is needed Can be performed at remote sites FDA approved 100µl whole blood sample/test Good correlation with standard flow assays | No CD4 percentage hence could not be used in infants and children Low throughput Moderate cost/test Labor intensive | 2000 | >8 |
| Dynal Manual Dynabeads assay | CD4 absolute count | Simple: fluorescence microscope , but can be adapted to light microscope can be performed at remote sites 125µl whole blood sample/test | No CD4 percentage hence could not be used in infants and children Low throughput Moderate cost/test Labor intensive Time consuming sample preparation Specific magnet and rotating mixer needed Less accurate at higher CD4 counts (>500) | 2,000-10,000 | 4-5 |
| Microchip-based systems | Absolute CD4 count | No technical skill required Robust Useful in remote places Can be upgraded with CCD camera for image analysis at distant central laboratory | Still under development Cannot give CD4 percentages | Still to be calculated | Still to be calculated |

| System | Parameters measured | Advantages | Disadvantages | Cost of the equipment US\$* | Cost/unit test* |
|----------------------------------|---|--|---|-----------------------------|------------------------|
| Semi-Bio manual slide technology | Absolute CD4 count | No technical Skill required Robust Useful in remote places | Still under development Cannot give CD4 percentages | Still to be calculated | Still to be calculated |
| Total Lymphocyte Count | WBC count and diff Lymph count and percent | Automated system Low cost Low expertise required | Correlation to clinical assessment of drug efficacy unknown Needs fresh sample for analysis to ensure accuracy | | < 1 |

*: The cost is subject to change with time

Chapter 6

Quality management in CD4 T lymphocytes enumeration

Quality management is a set of measures taken to ensure that defective products or services are not produced. Quality management incorporates the principles of quality control, quality assurance and quality improvement. In any laboratory dealing with HIV, quality is an essential factor to ensure that the overall quality of the results from the services or products is reliable, reproducible, traceable, and auditable. There are two key requirements for quality management of CD4 T lymphocytes testing. These include management and technical requirements.

6.1 Management requirements

The quality system has the following five key elements:

- Organizational management and structure
- Referential (quality) standards
- Documentation
- Assessment (Monitoring and evaluation)
- Training

Organizational management and structure

The overall responsibility for the design, implementation, maintenance and improvement in the quality system rests with the laboratory management. Quality is the responsibility of all the staff members of the organization. However, the top management needs to make a firm commitment to ensure quality and allocate adequate resources. Quality policy reflects the intention and commitment of the organization to attain quality. The policy can be implemented through a quality plan which, along with the policy, needs to be documented in the form of a quality manual.

Laboratory management delegates responsibility and authority to appropriate individuals who are directly responsible for implementing the quality policy and quality system and provides them adequate resources to efficiently discharge their duties. The inter-relationship between various staff members and their job description are decided by the management.

The management also makes all decisions and strategies with quality as the overarching theme. If the size of the organization requires it and the resources are available, a Quality Manager can be appointed to supervise and guide all activities related to implementation of quality. In smaller laboratories, one of the staff members can be given the additional responsibility of a “quality manager”.

Referential (quality) standards

The referential standards are an integral part of the quality system. These aim at ensuring safety and consistency. These need to be followed to meet the regulatory requirements as well as to monitor of the functioning of the laboratory.

Both management and technical standards need to be followed to ensure quality. These must also conform to the local laws.

Documentation

A document is a record of any information or instructions including policy statements, quality manuals, procedures, specifications, calibration tables, reports, job description, documents of external origin such as regulations, standards and examination procedures etc. These may be in various media, including hard copy or electronic.

The quality system of the laboratory shall define, document and maintain procedures to control all documents and information (from internal and external resources). The current version of relevant documents shall be available at all locations where operations needed for effective functioning of the quality system are performed.

Monitoring and evaluation

The laboratory management shall develop and implement quality indicators to systematically monitor and evaluate the laboratory’s contribution to patient

care. When the programme identifies opportunities for improvement within the system, the laboratory management shall take appropriate steps to address them. Error management shall be vigorously implemented.

Assessment of quality through audits (internal or external) and participation in external quality assessment schemes are other tools, the results of which should guide the management in further improving the quality.

Training

The quality system is only as good as the staff who actually work with it. No matter how good the quality system is on paper, if it cannot be translated into practice, quality cannot be achieved. Training must also include an understanding of why quality is important. Training should be competency based and must be followed by post-training support.

6.2 Technical requirements

Technical requirements refer to several key elements as follows:

- Personnel
- Infrastructure and environment conditions
- Laboratory equipment
- Pre/during/post-testing procedures
- Quality assurance
- Reporting of results

Personnel

Laboratory personnel are the most important resource in a quality management system. Laboratory management shall have an organization plan that includes personnel policies including the chain of command and job descriptions that define the qualifications and tasks of all personnel. Laboratory personnel shall possess appropriate education and have sufficient experience to perform the assigned duties. The laboratory should maintain a

record of job descriptions, educational qualifications, CVs and the training level of all personnel. The number of personnel shall be sufficient to perform the work required and to carry out other functions of the quality management system. Any quality system should be based on making laboratory personnel responsible for providing best quality in service. There should be a continuing education programme for staff at all levels.

Infrastructure and environment conditions

The laboratory should be spacious enough to support its workload without compromising the quality of work, quality control procedures, and the safety of personnel or patient care services. The facilities should be designed to ensure efficient operations, and comfort for its occupants. Similar provisions should also be made available for other related facilities such as sample collection and examination at sites other than the permanent laboratory.

Laboratory equipment

The laboratory shall be furnished with all necessary equipment required for the provision of services. All equipment shall be in working condition with manufacturer's instructions and operator's manuals. To ensure accurate and reliable performance, a strict programme for validation, calibration and maintenance should be in place. There should also be a documented and recorded programme for preventive maintenance recommended by the manufacturer. The operators should be trained in using the equipment. Whenever the equipment is returned after repairs, the laboratory should ensure that it is checked, validated and functioning satisfactorily before use.

Pre-/during/post-testing procedures

The accuracy of CD4 testing results depends on the quality of the blood sample submitted to the laboratory. In pre-testing procedures, blood samples should be properly collected, labeled, stored, packaged, and transported to the laboratory. Criteria or instructions should be developed and documented for acceptance or rejection of primary samples. These criteria should be strictly followed to ensure blood sample integrity.

During testing procedures, the laboratory should use the standard procedures which meet the needs of the users and are suitable for each laboratory setting. Preferred testing procedures are those that have been

published in the international guidelines, peer-reviewed texts or journals. Any alternative procedures or any in-house, adopted or modified procedures selected for use should be appropriately evaluated and validated in parallel with the standard procedures before being used for testing. There should be certified Standard Operative Procedures for each laboratory operation. They should be easily accessible to the staff who must adhere to SOPs strictly. Annexure 3 gives the list of SOPs that can be followed in a CD4 estimating laboratory.

In post-testing procedure, the laboratory director or manager should review the results of testing before reporting the results. Storage of blood samples should be in accordance with the laboratory policy. Disposal of blood samples no longer required should comply with bio-safety regulations.

Quality assurance

Quality assurance is a set of activities designed to ensure that the development and/or maintenance process is adequate to ensure the reliability and value of the testing data. Several sets of guidelines addressing quality assurance of CD4 testing have been developed. It is important that accurate Internal Quality Assessment Scheme (IQAS) and proficiency test or External Quality Assessment Scheme (EQAS), if applicable, be employed daily. IQAS shall be used to monitor both sample processing and instrument performance within the laboratory, whereas EQAS provides an assessment of the entire testing process, including pre- and post-testing procedures. However, participation in most of the current international external quality assurance programmes requires substantial funding to cover the cost of participation fees and carriages. There are also free external quality assurance programmes. In spite of their irregular schedules, they are useful and cost saving. Quality systems of the laboratory for CD4 testing shall include: 1) Laboratory bio-safety, 2) Methods for collecting; handling; transporting; storing and processing blood samples, 3) Quality control of reagents, 4) Instrument performance; quality control and maintenance, 5) Sample analyses, 6) Process for reviewing and reporting results, 7) Training programme for employees, and 8) Participation in the proficiency testing programme or external quality assurance programme.

Reporting of results

The laboratory management should be responsible for formatting reports. The laboratory director or manager should systematically review, evaluate

and authorize the results of testing. The report shall be clear and unambiguous and include date, time, procedure used, identification and location of the patient. It should also include identification of the person authorizing the release of the report with signature. The laboratory should retain all primary files, worksheets, and report forms for two years or in accordance with the approved policy. All data shall be reported in terms of percentages and absolute counts of CD4 T lymphocytes, or %CD4 T lymphocytes according to the equipment used (for eg: Only CD4 T lymphocytes counts in case of FACSCount). Percentages can only be reported if available from the testing performed. Where applicable, a set of normal range or reference intervals shall also be included in the report. It should be noted that the absolute counts are used in adult HIV-infected patients, whereas only the percentages are used in paediatric HIV-infected patients.

In summary, the laboratory should ensure that the Internal Quality Assessment (IQA) and External Quality Assessment (EQA) are in place. The essential elements of the IQA include personnel, instrumentation, document control, reagent control and corrective action on the problems encountered whereas the EQA includes participation in proficiency programme.

Chapter 7

Establishing a laboratory for CD4 T lymphocytes enumeration

Establishment of an optimal infrastructure for CD4 T lymphocytes count assay with reliable quality is one of the most important components for scaling-up the ART roll-out programme for monitoring disease progression and evaluating response to ART. However, the specifications required for setting up a CD4 T lymphocytes count testing laboratory need not be made exclusive and may be established in the HIV testing laboratories in the peripheral and intermediate levels. This chapter identifies the minimum requirements for all three levels.

7.1 Suggested requirements at different levels

Central or regional level with high work load

Although the high-throughput (up to 200 specimens per day) flow cytometer instruments (e.g. Becton Dickinson FACSCalibur and the Beckman Coulter EPICS XL/MCL) are expensive and technically more complex to maintain in developing countries, they are being used in major cities with well-developed central laboratories and where the workload is also usually high and there is better access to the timely technical support and consumables. Implementation of the sufficiently-evaluated alternative approach (e.g. Panleukogating strategy with limited monoclonal antibodies) using these high throughput instruments have been found to be workable and cost-effective. Currently, the commercial kit is available from Beckman Coulter Inc., USA.

Intermediate or district level with moderate workload

Some modified flow cytometers (FACSCount, Guava EasyCD4, CyFlow CD4 and PointCare) are considered to be medium-throughput (50 – 100 specimens per day) and designed for use in resource-limited settings. However, a few of the recently-introduced instruments need to be sufficiently evaluated before they are used. Also, some of these instruments do not have compatible external quality assessment panels.

Peripheral or remote Level with little workload

Some approaches are currently available for CD4 T lymphocytes count in settings where the throughput is low (less than 20 specimens per day). When the level exceeds 20 specimens per day, some of the medium-throughput methodologies could be considered. At present the peripheral laboratories should consider using of manual methods or shipping the blood sample to the district or central laboratory after stabilizing the sample.

7.2 Selection of methodology for CD4 T lymphocytes counts

The methodologies currently available for CD4 T cell count are based on distinct principles and have different operational characteristics. For selecting a suitable methodology, refer to Chapters 4 and 5.

7.3 Suggested testing at different levels

Samples can be processed at different clinical laboratory levels as per the different volume of samples/day. The choice could also depend on the availability of FCM, if not, simple total lymphocyte count or one of the alternative light microscope staining and counting assays could be used (Table 7.1).

Table 7.1: Guidelines on appropriate assays for different clinical laboratory levels

| Laboratory level | Number of specimens/day | Assay |
|-------------------------|--------------------------------|--|
| Peripheral | Less than 20 | Manual Assays (Cytosphere or Dynal) |
| | Less than 20 | Blood Stabilizer /transport to Central lab |
| | 20 - 50 | Medium-throughput FCM |
| Intermediate | 50 - 100 | Medium-throughput FCM |
| | 50 – 100 | High-throughput FCM |
| Central | Up to 200 | High-throughput FCM |
| | Up to 200 | High-throughput FCM |

7.4 Establishment of a CD4 T lymphocytes enumeration laboratory

The basic requirements of the laboratory are adequate space and work area, proper electrical conditions, storage facility for samples as well as reagents and documents. Table 7.2 gives details of these requirements at the three laboratory levels.

Table 7.2: Minimal infrastructure requirements for various levels of laboratory

| Requirement | Peripheral | Intermediate | Central |
|-------------------|---|--|---|
| Space | At least one room (approximately 3 m x 3). The room is divided by synthetic curtain into two for paperwork and test area. | At least two rooms (approximately 3 m x 3 m each) Room 1 for: <ul style="list-style-type: none"> • Paperwork and records. • Report preparation. • Supplies storage. Room 2 for: <ul style="list-style-type: none"> • Instrument placement. • Specimen processing. • Specimen storage. • Temporary disposal of bio-waste. | At least two rooms (approximately 4 m x4 m each) Room 1 for: <ul style="list-style-type: none"> • Paperwork and records. • Report preparation. • Supplies storage. Room 2 for: <ul style="list-style-type: none"> • Instrument placement. • Specimen processing. • Specimen storage. • Temporary disposal of bio-waste. |
| Major Instruments | <ul style="list-style-type: none"> • Light Microscope (Binocular). • Cell counter. • Hemocytometer. • Magnetic Device (If Dynal is used). • Refrigerator. • Blood mixer. • Micropipette-Reverse. • Protective equipments. | <ul style="list-style-type: none"> • Medium or high throughput flowcytometry depending upon the sample load • Air conditioned • Haematology Analyzer* • Refrigerator. • Blood mixer. • UPS 2-5 KVA • Micropipettes Reverse. • Protective equipments. | <ul style="list-style-type: none"> • High throughput FCM. • Haematology analyzer. • Air conditioned • Refrigerator. • Blood mixer. • UPS 5 -10 KVA. • Micropipettes-Reverse. • Protective equipments. |
| Manpower | Laboratory Technician-1 Laboratory Attendant-1 | Laboratory Supervisor-1 Laboratory Technician-2 Laboratory Attendant-1 | Laboratory Supervisor-1 Laboratory Technician-3 Laboratory Attendant-2 |

| Requirement | Peripheral | Intermediate | Central |
|--------------------|---|--|--|
| Other Requirements | – | <ul style="list-style-type: none">• Dust-free environment• Stabilized power supply. | <ul style="list-style-type: none">• Dust free environment• Stabilized power supply. |
| Comments | May be established in Local VCTC / Primary Health Centre. | May be established in district hospital | |

*: required only if a dual platform assay is used.

Annex 1

Collection and transport of specimens

Quality laboratory results begin with proper collection and handling of the specimen submitted for analysis. Correct patient preparation, specimen collection, specimen packaging and transportation are of vital importance. All necessary biosafety precautions should be followed during collection and transport of the blood samples. The Standard Operating Procedures (SOPs) should be available and should be followed strictly. Errors during these procedures can lead to serious consequences and affect patient and on subsequent patient management.

The collection procedures may vary depending upon the methodology used for the enumeration of absolute CD4 T lymphocytes, although Ethylenediamine tetra-acetate (EDTA) is generally recommended by most of the manufacturers. For dual platform approach the whole blood collected in EDTA should reach and be processed in the laboratory for lymphocyte counts within six hours.

Blood collection procedures

- Ensure that the specimen, collection tube is labeled with the date, time of collection, and a unique patient identifier/name. Labelling should be done legibly with a ballpoint pen or permanent marker.
- Record the age and sex of the patient and the collector information (name and initials) in the requisition form/work sheet, which should accompany the specimen being transported to the testing laboratory
- Collect blood specimens by venipuncture using evacuated tubes (e.g. BD Vacutainer blood collection tube) containing an appropriate anticoagulant, completely expending the vacuum in the tubes. Do not use tubes after the expiry date.
- Draw blood as per the standard procedure for venipuncture blood collection. The plastic collection tubes should be preferred due to the risk of breakage and possibility of cuts due to sharp edges with

the glass tubes. Partial draws (less volume in higher capacity tube) are not recommended, as the anticoagulant is hypotonic.

- Mix the blood well with the anticoagulant (invert the tube at least eight times) soon after the collection to prevent clotting.
- Collect all specimens from individual patients, wherever possible, at the same time of day (either morning or evening) to minimize diurnal variation
- Examine the tubes for sample integrity before transporting to the testing laboratory. Repeat specimens may be requested, if there is evidence of gross haemolysis or clots. Keep the specimen at room temperature (preferably below 28 °C) and do not refrigerate or freeze. Do not remove the stopper at any time.

Transport of whole blood specimen

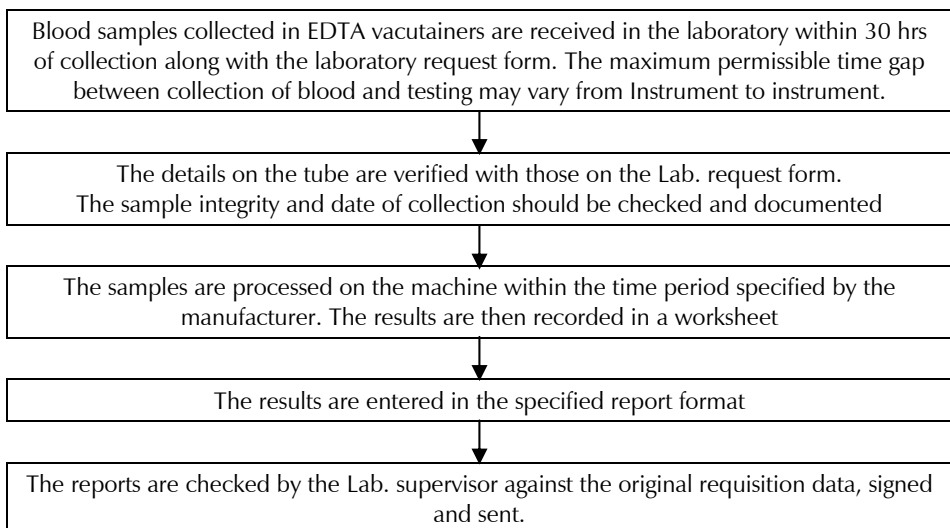
The Transportation of Dangerous Goods Act and the International Air Transport Association (IATA) dangerous goods regulations regulate the shipping of specimens containing infectious agents. HIV is classified as an infectious class 6.2 substance under the United Nations (UN) no. 2814. Under proper labelling instructions, the packaging must adhere to UN class 6.2 specifications. Packaging and transportation of HIV-infected material should be in accordance with the local regulations of the postal and/or courier service used. It is important to maintain and transport specimens at room temperature (approximately 18-22 °C). Avoid extreme temperatures. Temperatures greater than 37 °C may cause cellular destruction and affect both haematology and flow cytometry results. If the external temperature is high, the samples should be shipped with a cool (not refrigerated) pack.

The insulated container should be used for packing of samples keeping the cool packs separately. Use overnight carriers with an established record of consistent overnight delivery to ensure arrival the following day so that the specimens reach the testing laboratory within 24-30 hours. Check the equipment specifications to determine the acceptable time interval between collection and processing of the samples. During the transportation, the mechanical shaking should be avoided, as this may damage the cells integrity.

The steps given below should be followed for proper packaging.

- Make sure that the stoppers are tight and there is no breakage or leakage in the tube containing the specimen.
- Place the tubes in a leak-proof container (e.g., a sealed plastic bag) and pack this container inside a cardboard canister/box containing sufficient material (gauze or absorbent cotton) to absorb all the blood in case of breakage or leakage and also to avoid shaking of the tubes.
- Cap the canister/box tightly and seal it with self-adhesive tape.
- The request slip should be placed in another sealed plastic bag and fixed outside this canister/box with a rubber band.
- For mailing, this canister/box should be placed inside another canister/box containing the mailing label and biohazard sign.
- In hot weather, pack the specimen in an insulated container. If necessary, place this container inside another containing an ice pack and absorbent material. This method helps to retain the specimen at ambient temperature.

Overview of the sample receiving and processing in CD4 T lymphocyte testing laboratory



Annex 2

General bio-safety guidelines for immunophenotyping laboratory

The following safety practices should be adhered to:

- Never eat and drink in a laboratory.
- Hepatitis B vaccination should be given to all laboratory personnel and properly documented.
- Wear laboratory coats and gloves when processing and analyzing specimens.
- Never pipette by mouth.
- Never recap, bend, or break used needles – but recapping may be done with single hand method.
- Dispose needles and syringes in puncture-proof containers designed for this purpose.
- Handle and manipulate infectious fluid specimens with protective equipments such as a face shield, mask, goggles etc.
- Wash hands with soap and water after working with specimens in the laboratory and when leaving the laboratory.
- For stream-in-air flow cytometers, follow the manufacturer's recommended procedures to eliminate the operator's exposure to any aerosols or droplets of sample material.
- Disinfect flow cytometer wastes. Add a volume of undiluted household bleach (1% sodium hypochlorite) to the waste container before adding waste materials so that the final concentration of bleach will be 10% (0.1% sodium hypochlorite) when the container is full (e.g., add 100 ml undiluted bleach to an empty 1000-mL container).

- Disinfect the flow cytometer as recommended by the manufacturer. One method is to flush the flow cytometer fluidics with a 1% sodium hypochlorite solution for 5-10 minutes at the end of the day, and then flush with water or saline for at least 10 minutes to remove excess bleach, which is corrosive.
- Disinfect the blood spills with 10% sodium hypochlorite.
- In case of a large number of samples, the use of a flow cytometer equipped with an autosample loader/biosampler is recommended to minimize sample handling.
- Specimen preparation should be performed in a designated area, which should be thoroughly decontaminated after use.
- Management of exposure: In case of needle prick injury or contact of skin lesions with infected sample:
 - Report it to Biosafety committee.
 - Allow the site to bleed.
 - Wash it with plenty of soap and running water.
 - Determine HBsAg and HIV status of the source.
- Accordingly, HBsAg/ vaccine against HBV (if not previously immunized) / PEP for HIV should be started.
- Protection from LASER: Most flow cytometers contain low-power lasers, which are fully contained. Stream-in-air flow cytometers, mostly cell sorters, may contain high-power lasers not fully enclosed. Safety goggles should be worn whenever the laser beams are exposed.

Annex 3

Pipetting techniques

To obtain an accurate and precise enumeration of absolute CD4+ T cells, an accurate measurement of blood is required. Reverse pipetting is recommended. Reverse pipetting is only possible with air displacement pipettes. The following procedure describes good practice in pipetting:

- Press the operating button to the second stop.
- Dip the tip into the solution to a depth of 1 cm and slowly release the operating button. This action will fill the tip with a volume that is larger than the set volume. Wait 1-2 seconds and withdraw the tip from the liquid, touching it against the edge of the reservoir to remove excess liquid. (Do not touch the side of the tube if the same tip is going to be used for dispensing the same reagent in multiple tubes).
- Dispense the liquid into the receiving tube by pressing the operating button gently and steadily to the first stop. Do not immerse the tube deep into the liquid while dispensing. This volume is equal to the set volume. Hold the button in this position. Some liquid will remain in this tip and should not be dispensed.
- The liquid remaining in the tip can be pipetted back into the original solution or disposed together with the tip.
- Release the operating button to the ready position.

Important precautions

- Set the volume only within the range in the variable pipettes.
- Tips are designed for single use only.
- Avoid turning the pipette on its side when there is liquid in the tip.
- While pipetting, hold the pipette exactly in upright (vertical) position for accuracy. The tip should not be immersed in depth.

- Wipe the tip against the edge of the tube to remove excess liquid outside the tip before dispensing.
- Always store the pipette in an upright position when not in use.
- Maximize the volume of the variable pipette when it is not in use.
- Regular calibration is required and the recommended frequency is 3–6 months.
- The precision and accuracy of the pipette should be checked monthly.

Testing pipetting precision

The precision of pipetting should be evaluated periodically to ensure the accuracy of results. Retain all records of this evaluation procedure for quality assurance purposes.

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

Testing pipetting accuracy

The following procedure can be used to test the pipette and how accurately it measures volume. For calculation, a correction factor for the specific gravity of blood needs to be used if blood is used for the calibration. For this reason, distilled water is used because the weight of 1 μL of water is 1 μg .

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

Annex 4

Format for sample transport, receipt and report form

A Sample transport and receipt form

| Patient ID | Age | Sex | Date of Blood Collection & Time | Signature of Phlebotomist | Receiver's signature at the testing laboratory | Date of Blood sample receipt & Time | Integrity of the blood sample on receipt |
|------------|-----|-----|---------------------------------|---------------------------|--|-------------------------------------|--|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

B Reporting format

The following content must be included in the reporting results:

Laboratory Name and Address

Patient Name/ID#

Sex

Age

Referred by

Lab. Specimen Accession #

Date & Time of Specimen Collection

Reporting Date

Testing Method

Results

Reference Range

Authorized Signature

The Template of the report format is given below

Reporting format

Name and address of the Institute

Department of testing laboratory

No. assigned by the institute

No. assigned by the Clinic

Referred by

Date of collection.

Time of Collection (24 hours): .

Results of Immunophenotyping

| Parameter | Value | Reference Ranges* | |
|--------------------|-------|-------------------|--------|
| | | Male | Female |
| Absolute CD4 Count | | | |
| CD4 Percentage | | | |
| Absolute CD8 Count | | | |
| CD8 Percentage | | | |
| CD4:CD8 ratio | | | |

Instrument used:

Remarks:

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

- a) Sample was received in the laboratory after 24 hours of collection / clotted / hemolyzed.
- b) Abnormally low or high values.
- c) Others.

Date of reporting:

Laboratory-in-charge:

*: The reference ranges available at the national level should be used. If such data are not available it is recommended that attempts should be made to obtain national data.

Annex 5

Useful references

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Annex 6

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