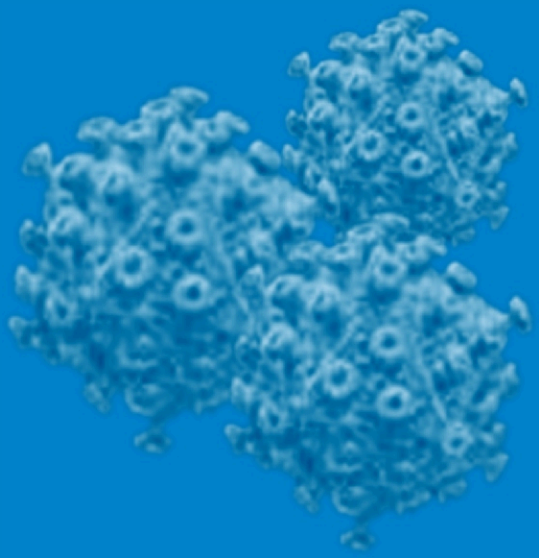
Revised Version 2009



Guidelines for HIV Diagnosis and Monitoring of Antiretroviral Therapy

World Health Organization
Regional Office for South-East Asia

SEA-HLM-382 (Rev. 2)
Distribution: General
Revised Version 2009

Guidelines for HIV Diagnosis and Monitoring of Antiretroviral Therapy

World Health Organization
Regional Office for South-East Asia

Printed in India

Contents

Annexes

Acronyms and abbreviations

AIDS	acquired immunodeficiency syndrome
ALT	alanine aminotransferase
ART	antiretroviral therapy
ARV	antiretroviral
CD4	CD4 T lymphocyte
CMV	cytomegalovirus
CNS	central nervous system
DOT	directly observed therapy
ELISA	enzyme-linked immunosorbent assay
EQAS	external quality assessment scheme
FBC	full blood count
GCLP	good clinical laboratory practice
Hb	haemoglobin
HIV	human immunodeficiency virus
IDU	injecting drug users
IFT	immunofluorescence test
LA	latex agglutination
MTCT	mother-to-child transmission (of HIV)
NGO	nongovernmental organization
NNRTI	non-nucleoside reverse transcriptase inhibitor
NsRTI	Neucleoside Analogue Reverse Transcriptase Inhibitor
NtRTI	Nuncleotide Analogue Reverse Transcriptase Inhibitor
OIs	opportunistic infections
PCR	polymerase chain reaction
PEP	post-exposure prophylaxis
PI	protease inhibitor

QA	quality assurance
QC	quality control
RT	reverse transcriptase
RTV-OU	ritonavir-boosted protease inhibitor
SOP	standard operating procedures
TB	tuberculosis
TLC	total lymphocyte count
UN	United Nations
UNAIDS	Joint United Nations Programme on HIV/AIDS
VCT	voluntary counselling and testing
WBC	white blood cells
WHO	World Health Organization

Preface to First Edition

HIV/AIDS is part of one of the greatest health crises ever faced by humanity. This pandemic has already killed 20 million people. Today, 40 million people are living with HIV. Each year, three million die of HIV/AIDS. However, most of these deaths could be prevented if they had access to antiretroviral therapy (ART).

In September 2003, WHO declared that failure to provide antiretroviral therapy to patients in developing countries will lead to a global public health emergency. Accordingly, WHO in collaboration with the Joint United Nations Programme on HIV/AIDS (UNAIDS) and partners set a target of providing three million people in developing countries with antiretroviral treatment by the end of 2005 (called the "3 by 5" Initiative). While this is an interim target, the long-term goal is of universal access to ART for all those who need it.

The primary objective of antiretroviral therapy is to prolong the survival, as well as improve the quality of life, of people living with HIV/AIDS. By bringing down the HIV viral load to sustained undetectable level, it is expected that ART will contribute also to HIV prevention.

Laboratory support is critical in all the areas of HIV diagnosis and management. Diagnosis of HIV infection cannot be established by any means other than blood tests by the laboratory. CD4 lymphocyte count is a prerequisite for the initiation of antiretroviral therapy and for monitoring treatment outcome. Both immunological and microbiological monitoring of antiretroviral therapy is, therefore, exclusively dependent on an efficient laboratory service.

While laboratory support to AIDS programmes is very important, the infrastructure, expertise and networking require strengthening in most countries of our Region.

In order to assist Member countries in building laboratory capacity, WHO has developed the Regional Guidelines on HIV Diagnosis and Monitoring of Antiretroviral Therapy. WHO is committed to provide all possible technical support to the countries, including for the purposes of strengthening laboratory support. I sincerely hope that these Guidelines will be helpful to Member Countries in scaling up ART and responding to the rapidly evolving HIV/AIDS epidemic.

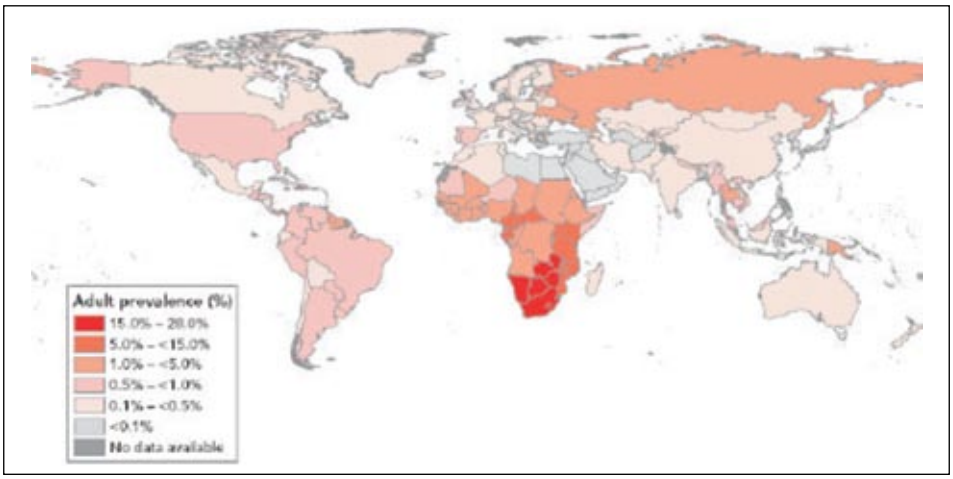
Dr Samlee Plianbangchang
Regional Director

1

HIV and laboratories: An overview

The human immunodeficiency virus (HIV) has changed the social, moral, economic and health fabric of the world in a short span. Today HIV/AIDS is the greatest health crisis faced by the global community. As per the 2008 UNAIDS Report this pandemic has till date killed nearly 25 million people. More than 30 million people are living with HIV, and an additional 7400 are added to this pool every day. It is expected that, if not treated, three million people will die of HIV/AIDS every year. It is estimated that of the millions of people living with HIV/AIDS (PLWHA) in developing countries, 6.7 million people require antiretroviral therapy (ART). Most of these are in 34 high-burden countries of Africa and Asia.

Figure 1: **A global view of HIV infection**
33 million people [30–36 million] living with HIV, 2007



HIV/AIDS in the South-East Asia Region

It is estimated that more than 4.2 million people are living with HIV in the countries of the South-East Asia Region. India ranks as the country with the second largest number (2.4 million) of such people in the world and is next only to South Africa. Four countries (India, Thailand, Myanmar and Indonesia) are considered high-burden countries. Currently, only 100 000 people with HIV in the countries of the Region are receiving ART, which is 31% of the number of people who need it.

The understanding of the transmission of HIV has given rise to various interventions which can prevent the occurrence of new cases. Reduction of viral load by efficient antiretroviral therapy is also a powerful tool in the overall interventions against HIV. To accelerate global efforts in augmenting antiretroviral therapy WHO is advocating and supporting the long-term goal of universal access to all those who need it. The basic principles are expanding access to ART and ensuring quality and adherence.

Role of the laboratory

Areas in which laboratories will play a critical role in implementing HIV programmes at the country level include detection of anti-HIV antibody as well as monitoring of ART.

Detection of HIV

The presence of HIV 1/2 infections in individuals can be ascertained only through the use of laboratory tests on body fluids such as blood, plasma, etc. The detection of HIV-2 has implications for ART . WHO and UNAIDS have established an algorithm for these of various tests for screening, surveillance and diagnostic purposes. These are being widely followed and their successful utilization has shown their utility.

Monitoring of ART

Monitoring of patients on chemotherapy is essential in all infectious diseases. It is of greater importance in cases of HIV because of the severity of illness, the potential of the virus to mutate and become resistant to drugs, and the

cost of treatment. Hence, laboratories are bound to play a critical role in the successful implementation of any ART programme. Various areas which need to be monitored are shown in Table 1.1: ART aims at reducing the viral load and augmenting the immune potential of the patient .

Table 1.1: Monitoring of disease progression and ART

Monitoring	Laboratory area
Virological	• Viral load • Resistance to antiretroviral drugs
Immunological and haematological	• CD4 T lymphocytes count • Total lymphocyte counts
Opportunistic infections	• Occurrence of new opportunistic infections • Recurrence of treated opportunistic infections • Antimicrobial susceptibility of bacterial pathogens • Reactivation of TB
Adverse drug reaction	• Liver and kidney function tests • Haematological parameters

HIV-1 viral load measurement has been found to be useful in monitoring treatment. It requires the establishment of a baseline plasma viral load before starting ART. The viral load in the case of successful ART becomes undetectable in four to six months of therapy. It can be measured using a variety of commercial kits. A number of home -brew assays for viral load are in use. However, these need rigorous standardization and continued quality assurance which often is not possible in a developing country setting. The assessment of viral load is a very expensive, complex and sophisticated procedure and hence it may be considered only when feasible.

Functions of laboratories

The laboratory techniques for supporting ART can be effectively applied for diagnosis and monitoring if a functional network of laboratories is created. Since many of the techniques are new, a network of laboratories with suggested functions at different levels is given in Table 1.2.

Table 1.2: Recommended tiered laboratory capabilities for diagnosis and treatment of HIV disease in resource-limited settings

Laboratory tests: Diagnosis and monitoring		Peripheral	Intermediate	Central
HIV antibody testing (Rapid and/or ELISA)		Yes	Yes	Yes
Haemoglobin		Desirable	Yes	Yes
Total lymphocyte count (TLC)		Desirable	Yes	Yes
Pregnancy testing		Desirable	Yes	Yes
Basic microscopy for TB and malaria (sputum smear for TB and blood film for malaria diagnosis)		Desirable	Yes	Yes
Full blood count (FBC)		No	Yes	Yes
CD4 T lymphocyte count		No	Yes (with flow cytometry or non-flow cytometry method)	Yes (with flow cytometry)
Liver and renal functions tests		No	Yes	Yes
Diagnostic tests for treatable HIV coinfections and major AIDS-related opportunistic diseases	Full cerebrospinal fluid (CSF) microscopy (including India Ink for cryptococcal meningitis), syphilis and other sexually transmitted infections.	No	Yes	Yes
	Diagnostic tests for other major treatable HIV coinfections and AIDS-related opportunistic diseases (hepatitis B virus, hepatitis C virus serology, bacterial cultures and diagnostic procedures for cryptococcosis , toxoplasmosis and other major OIs.	No	Desirable	Yes

Laboratory tests: Diagnosis and monitoring	Peripheral	Intermediate	Central
Full chemistry (including but not restricted to liver enzymes, renal function, glucose, lipids, amylase, lipase and serum electrolytes)	No	No	Yes
HIV viral load measurement and HIV drug resistance testing	No	No	Desirable
Participation in external quality assessment scheme	Yes	Yes	Yes

Support by WHO

HIV prevention and control programmes are being supported by WHO with globally coordinated activities. A strategic framework has been developed with the following five pillars:

- Global leadership, strong partnership and advocacy.
- Urgent, sustained country support.
- Simplified, standardized tools for delivering ART.
- Effective, reliable supply of medicines and diagnostics.
- Rapid identification and reapplication of new knowledge and successes.

One of the pillars of this initiative is to ensure the reliable supply of medicines and diagnostics. Realizing the inadequacies in the mechanisms at the country level to procure these, WHO has established AIDS Medicines and Diagnostic Services (AMDS) which will provide continuous technical support in procuring drugs and diagnostics.

2

Diagnosis of HIV infection

Objectives of HIV diagnosis

(1) Blood and blood products safety: This is achieved by mandatory testing of all donated blood units and blood products.

(2) Screening of donors of sperms, organs and tissues.

(3) Diagnosis of HIV infection in clinically suspected cases.

(4) Voluntary counselling and testing (VCT).

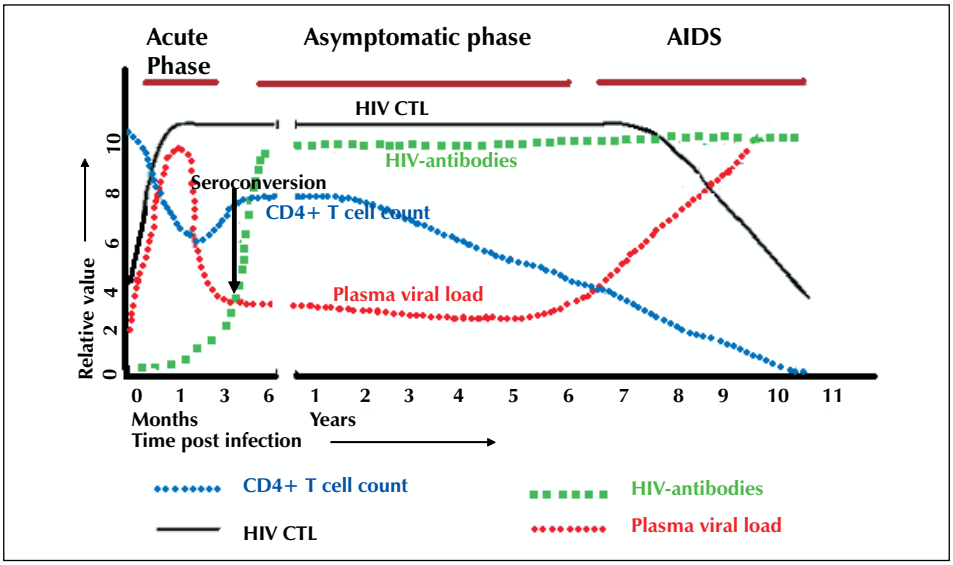
(5) Epidemiological surveillance using unlinked anonymous HIV testing. Here the result of the test cannot be linked with the identity of the person.

(6) Research and surveys.

For all individuals who are tested for HIV, the pre-test and post-test counselling should be an integral part of the HIV testing services.

In primary infection with human immunodeficiency virus, the virus in the blood can be demonstrated by nucleic acid-based test (PCR for pro-viral DNA and RT-PCR for viral RNA), p24 antigen testing or culture. Antibodies to HIV are detectable within four to six weeks of infection by commonly employed tests and in virtually all infected individuals within six months. Once antibodies appear in the blood, they persist for the lifetime (Figure 2.1).

Figure 2.1: **Laboratory markers during HIV-1 disease progression**



Diagnosis of HIV infection can be carried out by detecting any of the following:

- Antibodies to HIV
- P24 HIV antigen
- HIV nucleic acid (RNA/DNA)

The most commonly used method for the diagnosis of HIV infection is detection of anti-HIV antibodies in serum/plasma. It is economical, rapid and can be performed easily in most laboratories. HIV antibody assays are now commercially available in various formats. It is necessary to differentiate between HIV-1 and HIV-2 infections as the treatment varies for the two types. HIV-2 is intrinsically resistant to NNRTI drugs. Although some of the rapid tests can indicate the presence of anti-HIV-2 antibodies in a sample, the frequent occurrence of cross-reactions makes virus speciation on serological grounds problematic. Furthermore, some studies have reported high sensitivity (91% to 100%) and specificity (81% to 100%) for HIV-2 antibody testing by Western blot (immunoblot). On the contrary, over-estimation of the incidence of dual infections caused by HIV-1 and HIV-2 based on serological testing has been reported in India. Since the role of western blot is not conclusive in the diagnosis of HIV-2 infections, use of molecular techniques may be considered.

Detection of anti-HIV antibodies

ELISA is the most widely used technique for the detection of antibodies to HIV. HIV antibody tests have been classified as first- to fourth-generation tests based on the principle used in the assays as well as the type of antigens used.

Table 2.1: Generation of ELISAs.

Generation	Antigens/antibodies	Comment/characteristic
First	Antigens from HIV lysates	Lack of sensitivity and specificity
Second	Recombinant proteins and/or synthetic peptides	Improved sensitivity and production of combined HIV-1/ HIV-2 assays
Third	Use labelled antigen as conjugate	Very high sensitivity and able to detect IgM antibody; reduced the window period considerably
Fourth	Detection of both HIV antigen (p24) and antibody	Further reducing the window period

Enzyme Linked Immunosorbent Assay (ELISA): ELISA requires a washer and reader and is suitable for use in laboratories where the sample load is high. Using antigens employed in the third-generation ELISA systems, several rapid tests have been developed and are widely used. The commonly employed rapid anti-HIV tests are based on the principle of immunofilteration (flow through), immunochromatography (lateral flow), dot immunoassay, or particle agglutination (e.g. gelatin or latex). Rapid tests are visual tests that do not require the ELISA reader. These tests are available in smaller test packs and each device has procedural control system. Therefore, these are suitable for a laboratory that tests smaller sample numbers as well as for stand-alone sample. They are technically simple to perform and most of them have sensitivity and specificity comparable to ELISA. Moreover, most rapid test-kits can be stored at ambient temperature (20 °C to 25 °C) and the diagnostic performance is comparable to that of traditional ELISAs.

The cost of HIV antibody tests varies depending on the type of test used. ELISA-based tests cost between US$ 1–3 per test while rapid tests range from US$ 2–5 per test. The specimen of choice for anti-HIV antibody testing is serum or plasma. Assays for the detection of anti-HIV antibodies in whole blood, saliva/oral fluid, urine and dried blood spot have also been developed. However, these specimens (other than plasma or serum) should be subjected to testing only when their utility has been thoroughly validated.

HIV infection during window period can be detected by demonstrating the presence of virus and virus components. PCR and detection of p24 antigen may be helpful.

Diagnosis of HIV infection in children and infants

Diagnosis of HIV infection in babies born to HIV-infected mothers cannot be established by conventional antibody tests. The presence of anti-HIV antibody in the newborn may not necessarily indicate primary infection. It may be due to the presence of passively transmitted anti-HIV antibodies from the mother to the uninfected child. These maternal antibodies may persist in the infant for as long as 18 months. Hence, virological assays such as HIV DNA-PCR or HIV RNA assays represent the gold standard for diagnosing of HIV infection in children younger than 18 months. Some DNA and RNA PCR assays support the use of dried blood spots (DBS) samples, which have considerable advantages in settings where sample transportation and storage are problematic.

A definitive diagnosis of HIV-1 infection can only be made on the basis of two positive HIV-1 DNA or RNA assay results at six weeks after birth (sensitivity >98%). For infants born to HIV-1–infected mothers, it has been recommended that diagnostic testing with HIV-1 DNA or RNA assays be performed within the first 14 days of life, at one to two months of age, and again at three to six months of age. If any of these test results are positive, repeat testing is recommended to confirm the diagnosis of HIV-1 infection. However, if an infant is breastfeeding he or she remains at risk of acquiring HIV infection throughout the breastfeeding period and, therefore, negative virological test results can be assumed to reliably indicate HIV infection status only after six weeks after complete cessation of breastfeeding (Annex 9).

Alternatively, if the first PCR is negative in a non-breastfed baby, confirm with a second PCR test at six months. Definitive exclusion of HIV-1 infection is based on two negative virological test results, one obtained at one month of age and one obtained at four months of age, or two negative HIV-1 antibody test results from separate specimens obtained at six months of age. For exclusion of infection, the child should have no other laboratory or clinical evidence of HIV-1 infection and should not be breastfed.

Dried blood spot (DBS) represents a paradigm shift in accessibility to nucleic acid testing for HIV infection. Nucleic acids in DBS have been shown to be stable for several months at ambient temperatures, provided the DBS specimens have been thoroughly dried and stored with desiccant. Thus DBS specimens can be collected at remote rural sites and transported to a central or regional testing laboratory.

However, given the expense and complexity of nucleic acid testing, the World Health Organization strongly encourages the development of technologically simpler, less expensive assays that can be used to diagnose HIV-1 infection in early infancy. The ultra-sensitive p24 (Up24 assay) assay is gaining support as a tool for detection of HIV-1 infection in infants following mother-to-child transmission. The ultrasensitive p24 antigen assay is an ELISA and is, therefore, suited to health facilities where serologic al testing is routinely performed. The performance of the Up24 assay for HIV diagnosis in infants and young children has been evaluated in a number of studies in countries with different HIV subtypes yielding sensitivities and specificities ranging from 96% to 99% when compared to HIV DNA PCR testing.

By the age of 12 months most uninfected HIV-exposed children lose maternal antibodies and testing HIV antibody-positive at this age can be considered indicative of HIV infection. This should be confirmed by repeat antibody testing after the age of 18 months. If an HIV-exposed child between 9 and 18 months of age had never been breastfed or had stopped breastfeeding for at least six weeks and had a negative HIV antibody test result, then the child should be considered uninfected with no further testing required unless symptoms develop.

Specimen collection

Optimal time of specimen collection	Blood specimens can be collected at any convenient time
Correct specimen type and method of collection	Whole blood or anticoagulated blood DBS may also be collected. However, it is advisable to collect multiple spots for repeat assay or quality control
Adequate quantity	Approximately 3–5 ml

Specimen transport and storage

Time between specimen collection and processing	• Whole blood specimens should be transported at ambient temperature (20 °C to 25 °C) and processed as soon as possible or within 24 hours. • If serum/plasma has been separated, it can be stored in a refrigerator (2 °C to 8 °C) for a week or can be transported at 2 °C to 8 °C • For longer storage, store in a freezer at –20 °C

Criteria for rejection of sample

The following are the criteria for the sample rejection:

- Hemolyzed samples
- Samples showing turbidity
- Specimens not stored and transported properly
- Sample that does not carry appropriate label
- Samples that have leaked

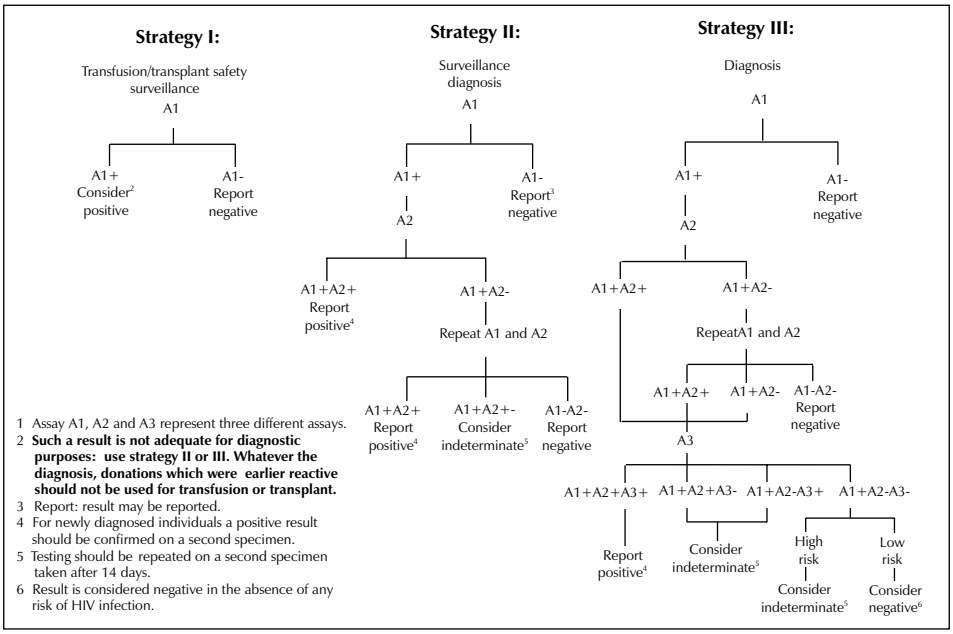
Table 2.2: UNAIDS and WHO recommendations for HIV testing strategies according to test objective and prevalence of infection in the sample population

Objective of testing	**Prevalence of infection in the category to which the patient belongs (percentage)**	Testing strategy applicable
Screening of blood and blood products	–	I
Surveillance	>10 <10	I II
Diagnosis: With clinical signs/ symptoms	>30 <30	I II
Asymptomatic	>10 <10	II III

HIV testing strategies

The strategies used for HIV testing are summarized in Table 2.2. The type of strategy to be adopted would depend on the ultimate purpose for which HIV testing is being carried out. Figure 2.2 outlines the HIV antibody detection algorithm that is used for the various HIV testing strategies. One of the essential prerequisites for the use of this algorithm is that the first, second and third tests (A_1,A_2 and A_3) employed are based on different immunological principles and/or use of different HIV antigens in the assay. The follow-up sample from patients with indeterminate result should be collected two weeks after the first sample collection. If the second sample also shows indeterminate result,

Figure 2.2: **Schematic representation of the UNAIDS and WHO HIV testing strategies**



1 Assay A1, A2 and A3 represent three different assays.
2 **Such a result is not adequate for diagnostic purposes: use strategy II or III. Whatever the diagnosis, donations which were earlier reactive should not be used for transfusion or transplant.**
3 Report: result may be reported.
4 For newly diagnosed individuals a positive result should be confirmed on a second specimen.
5 Testing should be repeated on a second specimen taken after 14 days.
6 Result is considered negative in the absence of any risk of HIV infection.

it should be tested by a confirmatory assay (e.g. Western Blot). However, if the confirmatory test fails to resolve the serodiagnosis, follow-up testing should be undertaken at four weeks, three months, six months and 12 months. After 12 months, such indeterminate results should be considered negative. However, the molecular assays (HIV-1 and HIV-2 NAT) can be used to resolve specimens giving repeated (>2 times) indeterminate results.

Reporting procedure

Report	Negative – if initial/screening test shows non-reactive result. Positive – if the sample shows reactive results concordantly by the three tests as per the algorithm. HIV-1 positive/HIV-2 positive or dual reactive as per the algorithm should be mentioned. In case of dual reactivity, confirmation by molecular assays may be considered. Indeterminate – if the sample shows discordant results by the three tests. The follow-up testing is recommended as mentioned above.

Selection of test kits

A wide variety of test kits are now available for HIV diagnosis. The selection of appropriate test kits/assays/reagents is critical to ensure quality in laboratory services. Every country or laboratory must, therefore, define a policy for selection, evaluation and procurement of the most appropriate kit. Selection of assays/reagents is a complex process that needs to be planned carefully. The overall performance of an assay/reagent depends upon a number of local factors. Quite often the manufacturer's quoted sensitivity and specificity figures may not reflect the actual working figures. Therefore, selection of an assay/reagent needs to consider the testing needs of a centre and the resources available to meet those needs. Procurement systems may have a significant impact on the selection of kits. Stock control is vital, especially where continuity of supply cannot be guaranteed. Ongoing monitoring systems are essential to identify problems either with the assay/reagent or the laboratory.

The steps that are involved in the selection of a test kit/reagent for a testing laboratory are:

(1) Sensitivity and specificity of the test: Sensitivity is the ability of an assay to identify all infected individuals (true positives). The specificity of an assay is the ability of an assay to identify uninfected individuals (true negatives). Kits/assays should have a sensitivity >99% for Rapid test/ELISA) and specificity >98%.

(2) Ease of testing, competency of the staff performing the test and infrastructure available.

(3) Type of sample: serum/plasma/whole blood/dried blood spot/other body fluid

(4) Type of test controls provided: Reliable kit systems that provide internal controls are preferred. Similarly, for rapid tests, kits that provide a sample addition check are generally preferred.

(5) Number of tests per kit.

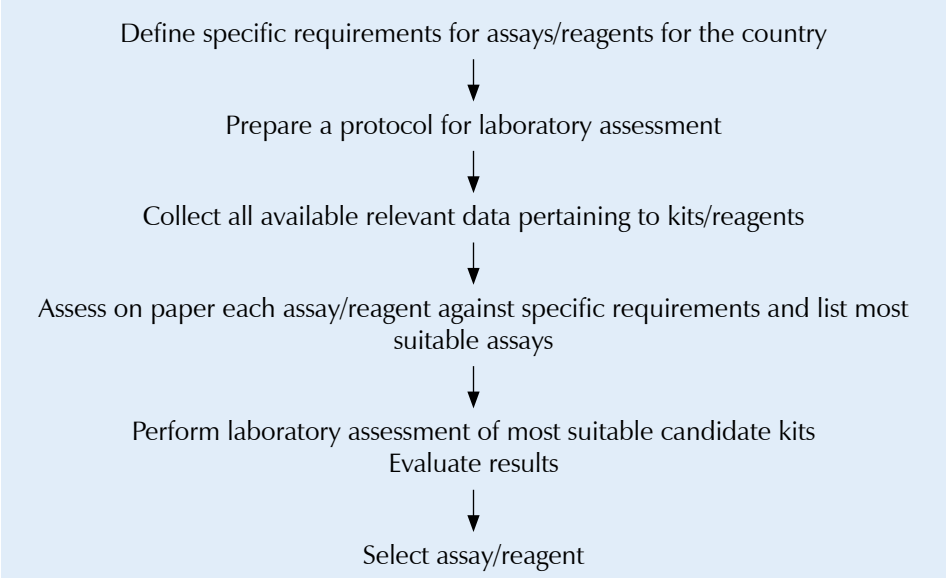
(6) Shelf life of kit as per the kit recommendation: Kits with a longer expiry date are preferred over those that have more recent expiry dates.

(7) Storage and handling requirements during transport and their feasibility.

(8) Resource availability: Finally, the choice of a test kit/reagent would depend a great deal on the availability of financial resources, existing systems in place in the laboratory and the time scale in which results are expected to be delivered.

(9) Time required to complete the test: If HIV results are required to be obtained within a short time and only a few samples need to be tested in a laboratory, rapid and simple HIV kits may be preferred over other assays. On the contrary, if a large number of samples are to be tested, assays such as ELISA may be preferred.

Evaluation and final selection of kit



Evaluation and procurement

Procurement of test kits/reagents depends on several factors which need to be considered. These factors in turn would depend on the logistics and practices that are prevalent in the laboratory, such as mechanism of procurement of kits, specific requirements of laboratory and time-frame for procurement.

Monitoring subsequent performance

Monitoring the performance of a reagent/kit is a continuous process which begins from the time of procurement until all the kits are used or reach their expiry dates. Each country should draw up a plan for periodic monitoring of reagents/kits at various testing levels in the country, much akin to those that are already existing for vaccines in many countries. It may be advisable to have periodic "post-marketing surveillance" of the kits carried out in the central laboratory which gets these kits for evaluation from various peripheral testing laboratories.

Technical support from WHO

Realizing the inadequacies in the mechanisms at the country level to procure reagents/kits, WHO has established a mechanism called AIDS Medicines and Diagnostic Services (AMDS) with the following features:

- AMDS is created to expand access to quality, effective anti-retroviral therapy (ART) by facilitating increased supply of drugs and diagnostics in developing countries.
- AMDS will provide to manufacturers information and forecasting about global demand/market.
- AMDS will provide to buyers sources, process and patents on drugs and diagnostics and assist them in obtaining the best prices for individual or pooled demand.
- AMDS will provide technical support to countries in improving their procurement mechanisms.
- AMDS will assist countries, NGOs and other non-profit organizations.

AMDS has also created a pre-qualification project (PQP) which aims at ensuring quality, safety and efficacy of HIV/AIDS medicines and diagnostics. It assesses products voluntarily submitted by manufacturers and certifies their conformation to WHO standards. The approved drugs and diagnostics are shown in the public domain. Twenty-three HIV kits are available at present. CD4 and viral load kits are also being monitored. These services are managed by WHO in collaboration with UNICEF, UNAIDS, UNFPA and the World Bank. AMDS, however, will not provide free drugs or diagnostics to the countries. All the information pertaining to AMDS can be accessed from the WHO website (www.who.int).

3

Immunological monitoring of antiretroviral therapy: CD4 T lymphocytes counts

What are CD4 T lymphocytes?

Cellular components of blood comprise red blood cells and white blood cells. Two populations of leucocytes constitute the latter — the granulocytes and non-granulocytes, including the lymphocytes. Surface receptors of the lymphocytes provide identity to sub-populations of lymphocytes which differentiate into unique clusters. This property gives the subtypes of lymphocytes a nomenclature of clusters of differentiation followed by the number of the unique subtype (CD1, CD2, CD3, CD4…). CD stands for cluster of differentiation; CD numbers are now used to identify cell surface antigens that can be distinguished by monoclonal antibodies. CD4 T lymphocytes (CD4+ T-cells), commonly known as T helper cells, play a vital role in maintaining the integrity of the human immune system.

Importance of CD4 T lymphocytes

A primary target of HIV is CD4 T lymphocytes which are preferentially depleted during the course of the disease. It is well recognized now that accurate and reliable enumeration of CD4 T lymphocytes is very crucial for monitoring the rate of progression to AIDS, both for initiating prophylaxis for opportunistic infections as well as monitoring the impact of antiretroviral therapy (ART).

Methods of enumeration of CD4 T lymphocytes

Immunofluorescence analysis by flow cytometry (FCM) is the gold standard for CD4 T lymphocytes measurements. FCM is the first choice, if large number of

specimens need to be tested. To obtain an absolute CD4 T lymphocytes count, two concepts (Annex 4 and 5) are utilized:

Dual-platform (DP) approach

The DP approach uses two instruments to generate absolute CD4 T lymphocytes: an FCM for generating a percentage CD4 T lymphocytes among lymphocytes and a haematological analyser to enumerate the absolute lymphocyte counts. An absolute CD4 T-cell count is derived by multiplying % CD4 T lymphocytes by the absolute lymphocyte count. Examples of these DP FCM instruments include BD FACSVantage, FACSCalibur/FASCScan/FACSort or Beckman-Coulter Epics XL/XL-MCL.

Single-platform (SP) approach

This technique enables absolute CD4 T lymphocytes counts to be derived directly without the need for a haematological analyzer, i.e., the use of volumetric counting (Partec CyFlow), microfluorometry (Guava) and, most commonly, the addition of reference fluorescent beads of a known density (eg., BD-Truecount and Coulter Flowcount) to the sample.

Other alternative methods

Flow cytometry is a widely used method for estimation of CD4 T lymphocytes. Flow cytometer and reagents are expensive and hence are a cause for concern in developing countries. For those countries and settings where the infrastructure is not available or difficult to set up for such FCM technologies, a number of alternative assays have been developed and most of them are commercially available. These assays have been evaluated against the gold standard method and reported in literature.

Total lymphocyte count

In cases where CD4 testing cannot be assessed, the presence of a total lymphocyte count (TLC) of 1200 cells/ul or below may be used as a substitute indication for ARV treatment in symptomatic HIV-infected patients. While the total lymphocyte count correlates poorly with the CD4 T lymphocytes count in asymptomatic patients, in combination with clinical staging it is still a useful marker of prognosis and survival.

There are limitations in the use of TLC as a substitute for CD4 T lymphocytes counts. For example:

- Lack of correlation with CD4 counts in asymptomatic patients
- Variation due to intercurrent infections or drugs.

Microscope-based CD4 counting systems

These alternative assays are, however, fairly labour intensive and thus less appropriate for a large number of samples. Moreover, qualified personnel are required for making accurate measurements.

- Microbead system (Dynal Biotech, Oslo, Norway) uses two types of beads. The first bead removes monocytes from the sample and the second (CD4) estimates CD4 T lymphocytes that get stained with acridine orange to make the cell nuclei visible for counting under a fluorescent microscope. The initial cost of equipment for a fluorescence microscope is low (US$ 6000) with a running cost of US$ 3–5 per test. A modified DynaBeads system with an alternate stain for the cells can be used with a light microscope.

Evaluations of Dynal bead assay was highly comparable with the standard FCM and found to be accurate and reproducible. However, this is a manual and labour-intensive assay. To scale these up to match expanding access to ART may prove a challenge. 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.

Affordable flow cytometry

Combined use of CD4 and CD45 conjugated antibodies using Panleucogating (PLG) methodology (eg. Beckman *Coulter FlowCARE CD4* Reagent) has been found to be workable and cost-effective.

Modified single platform volumetric flow cytometry

Cyflow from Partec

The system works on volumetric method, i.e. a known amount of blood with a single antibody reagent. It can also be run on solar panels and car batteries, and hence may be used in remote areas. The methodology is simple to carry

out based on an SP method with single antibody reagent and ten-minute incubation. The system showed good correlation with the CD4 T lymphocytes counts obtained by conventional flow cytometry. However, an experienced technician is required for accurate measurement. The CyFlow capital equipment costs approximately US$ 20 000 and the cost per test is US$2.

Guava technology

A SP system that uses CD3 antibodies to measure T lymphocytes and CD4 antibodies to estimate absolute T-cells expressing CD4. The system showed good correlation with conventional flow cytometry and is easy to operate. It uses smaller blood volume. It requires very minimal infrastructure facilities and it is easy to train technologists to perform the test. The cost per test is around US$ 2. However, the equipment costs about US$ 26 000.

The recent evaluation studies with these two modified flowcytometry systems have shown a good correlation with the standard FCM.

Selection of alternative methodology for CD4 T lymphocytes count

The following specifications should be considered for the selection of the better technology for CD4 T lymphocytes count:

- The equipment should be simple to operate, easy to maintain and require minimal training.
- Methodology needs minimal infrastructure laboratory facilities.
- Methodology should have the internal QC procedures.
- Test kits should be cost-effective and available anytime.
- Easy access to the technical specialist/service engineer support.
- Supplying company should be in a position to supply the critical parts of the equipment with short turnaround time.

For HIV/ART monitoring, all CD4 T lymphocytes estimation for the same patient must be done using the same technology and same laboratory to ensure comparability.

Utility of CD4 T lymphocytes count in monitoring

According to the WHO recommendation, HIV-infected adults should start ARV therapy when infection has been confirmed and one of the following conditions is present (Table 3.1):

Table 3.1: WHO recommendations for initiating ARV therapy in HIV-infected adults and adolescents according to CD4 T lymphocytes counts and total lymphocyte counts*

WHO Stage	CD4 T lymphocytes counts	Total lymphocyte counts
IV	Irrespective	Irrespective
III	<350 cells/uL	Irrespective
II	<200 cells/uL	<1200 cells/uL
I	<200 cells/uL	Not applicable

* It should be noted that in HIV-related symptoms (Stage II, III), a total lymphocyte count of 1200 cells/ul can be substituted for the CD4 T lymphocytes count when the latter is not available. However, asymptomatic HIV-infected patients (Stage I) need not be treated because the total lymphocyte count correlates poorly with the CD4 T lymphocytes count in asymptomatic patients.

Sample guidelines

Blood samples are collected by venipuncture into EDTA (preferably K2) containing tubes, mixed well and processed within the time-frame (the sample stability varies depending upon the equipment and the type of kit). For example, the samples should be processed within 48 hours (stored between 20 °C–25 °C) after the collection for the FACS Count. Blood samples should preferably be collected within a fixed time in the day (morning or evening) so as to avoid diurnal variations. Blood sample that is not suitable should be rejected. For example, blood sample that cannot be performed within the time-frame, or if blood sample is hemolyzed or frozen or clotted or is without proper labelling. Data on storage temperature for Partec instrument needs to be incorporated.

Suggestions for testing at different levels

Samples can be processed at different clinical laboratory levels and different volume of samples/day. FCM is the method of choice. If FCM is not available, simple total lymphocyte count or microscopy-based assays could be used (Table 3.2). However, these have major limitations.

Table 3.2: Guidelines on appropriate assay for different clinical laboratory levels

Laboratory level	Number of samples/day	Assay
Peripheral	1–5	Total lymphocyte count
	1–5	Dynabeads
	1–5	FCM CD4 testing*
Intermediate	5–20	FCM CD4 testing*
	5-20	Transfix**/CytoChex BCT***/ Central laboratory
Central	>20	FCM CD4 testing

* Modified or affordable flowcytometry methodologies could be considered after the required validation.
** Transfix is a whole blood stabilizing reagent from United Kingdom Quality Assessment Service (UKNEQAS). When Transfix is added to fresh blood, the sample retains its integrity for almost one week. The use of Transfix is therefore suitable to increase the catchment area of central laboratories. (***CytoChex BCT is also a blood stabilizing reagent used to delay blood sample degradation. It is a product manufactured by Streck, Inc. USA).

Gold standard for CD4 T lymphocytes count: Flow cytometry

Principle

Flow cytometer operates by introducing cells stained with fluorescence conjugated antibody or absorption dyes in a fluid stream under a slight pressure to pass through a nozzle into the beam of light, usually generated by a laser. Light that is scattered and emitted by cells is then separated into constituent wavelengths by a series of optical filters and mirrors. This separated optical light falls on individual photodetectors and is then translated into electrical pulse, or analog signals, proportional to the amount of incident light detected by the detectors. Each analog signal is finally converted into a digital signal. The magnitude of digital signals is then processed by the data processing and analysis unit. The numbers are proportional to the amount of light emitted from, or scattered by, individual stained cells.

Samples

Cells to be measured must be suspended in a liquid. So, ideally, this is simple for whole blood. Anticoagulant blood is stained with appropriate monoclonal antibody that binds to the specific antigens, i.e. CD4 that are to be measured. Normally, the monoclonal antibody is directly conjugated with fluorochrome.

Advantages and disadvantages

Table 3.3: Advantages and disadvantages of using flow cytometer for CD4 T lymphocytes testing

Advantages	Disadvantages
Reference method	Expensive instrument
High reliability	
High accuracy, precision and reproducibility	Expensive maintenance
Handling large number of samples	Need well-trained technician

Cost

At present, the cost of CD4 testing varies from country to country and ranges between US$ 12–US$ 30. However, the recent development of a simplified *panleucogating* method together with the use of generic monoclonal antibody reagents drastically cut the cost of CD4 test by more than one-fourth.

Quality system

Several sets of guidelines addressing quality control of CD4 T lymphocytes enumeration have been developed. It is important that accurate daily internal quality control and proficiency test or external quality assurance programmes be employed to ensure the reliability and value of CD4 T lymphocytes data. However, participation in most of the current international external quality assessment programme requires a substantial amount of funding to cover the cost of participation fees and carriages. There are free external quality assessment programmes, in spite of their irregular schedules; they are useful and cost saving.

Reporting result

All data are reported in terms of percentages and absolute counts of CD4 T lymphocytes, or %CD4 T. 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 the report, the mean and reference of both percentage and absolute CD4 T lymphocytes (normal ranges) should also be given. Each clinical laboratory should validate normal ranges.

Total lymphocyte counts

Principle

Total lymphocyte counts are easily obtained by automated haematological analyser on aspirated blood samples appropriately diluted with a solution (e.g., acid or detergent) that lyses the red blood cells but preserves leukocyte integrity. A typical automated haematological analyzer performs white blood cell counts by either impedance or light-scattering technology or both. Cells in suspension are made to flow through a small orifice across which an electric current is flowing as in impedance technology. As a cell enters the orifice, the flow current is reduced. Electronic circuits detect the decrease in current and thus the presence of the cell. In a light-scattering haematological analyzer, 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 since the volume flow rate can be measured one can thus determine the number of white blood cells per ul and or total lymphocyte count per ul of the blood sample.

Samples

Collect blood samples by venipuncture into tubes containing K_2EDTA anticoagulant. Mix the blood well to prevent clotting. Aliquot appropriate amount of blood into tube containing lysing buffer (e.g. an acid buffer or a detergent) to lyse red blood cells. The lysed whole blood samples are processed and analysed for their white blood cell count and total lymphocyte count by using haematology cell counter.

Advantages and disadvantages

Table 3.4: Advantages and disadvantages of using total lymphocyte count for ARV therapy

Advantages	Disadvantages
Inexpensive unit cost	High variation (CV up to 15%)
Simple and commonly available test	No EQAS
Less complicated instruments compared to FCM	Fault results due to nucleated red blood cells, platelet aggregation and nonlysed red blood cells.

Cost

The cost of total lymphocyte counts is cheap compared to CD4 testing. It varies from country to country but is around US$ 1–US$ 2.

Quality system

Currently no external quality assessment scheme on white blood cell counting is available. Only internal quality controls supplied by each manufacturer are recommended. It is important that accurate daily internal quality control and proficiency tests, if applicable, are employed to ensure reliability and value of the total lymphocyte count data.

Reporting result

In haematology cell counter the total lymphocyte count is calculated from direct measurement of lymphocyte count, and expressed as cells x 10^3/ul. Normal value for total lymphocyte count is 2.6 x 10^3/ul (range 1.0-4.9 x 10^3/ul). Normal ranges should be validated by each clinical laboratory.

More details on the enumeration of CD4 T lymphocytes enumeration can be found in the WHO-SEARO Guidelines (HLM-392).

4

Virological monitoring of ART

Viral load testing

HIV-1 viral load measurement is useful for monitoring treatment. A baseline plasma viral load is established before starting ART. Periodic monitoring is essential. It is predicted that with successful therapy a fall of 1.5 to 2 log in plasma viral load occurs within 4-6 weeks. With successful ART, it should become undetectable in four to six months of therapy.

Viral load is measured using a variety of commercial kits based on nucleic acid testing (NAT) and non-NAT (Annex 6 and 7). The Roche Amplicor HIV-1 monitor test, version 1.5, is used widely. The assay uses *gag* specific primers for the highly conserved region. The lower limit of detection with the standard assay kit is 400 RNA copies/ml and the upper limit is 750 000 RNA copies/ml. The Amplicor ultra-sensitive kit detects down to 50 copies/ml of plasma.

A real time PCR is being tested in some laboratories. The cheaper cost of the test and the advantage of avoiding batch testing are some of the plus points. The test uses primers and probe sets specific for the LTR region of the HIV genome, which is conserved across subtypes. The primers need to be selected carefully as some subtypes may be estimated in relatively lower copy numbers with some of the primers. The lower limit of detection is in the range 50 IU/ml. The virological assays are useful tools in monitoring for the emergence of resistance in HIV against antiretroviral drugs.

An important concern with viral load assays is the genetic diversity of HIV-1. In particular recombinations between several subtypes of HIV-1 have generated many circulating recombinant forms . Therefore, the impact on the sensitivity of currently available commercial viral load assays—most of which

are based on HIV 1 subtype B—and their appropriateness for use in countries where subtype C is not prevalent needs to be considered before choosing an assay.

HIV drug resistance assays

The increase in the use of ART is expected to lead to the emergence of drug-resistant mutants of HIV-1. This has been the experience from other parts of the world. The inherent mutability of the reverse transcriptase (RT) gene of HIV allows for drug resistance to emerge under selection pressure. The protease gene also undergoes mutations, which manifest as a failure to respond to protease inhibitors.

A survey of drug resistance of indigenous HIV strains is essential to ascertain the usefulness of the antiretrovirals, especially in public health programmes. Drug resistance should be suspected if the plasma viral load does not show a greater than 1 log fall within eight weeks of therapy.

WHO has prioritized the prevention, surveillance and monitoring of HIV drug resistance and has developed an approach to prevent the emergence and transmission of HIV drug-resistant virus. HIV drug resistance surveys are targeting untreated individuals, particularly patients who had recently become HIV-infected. Public health action would be essential if such a survey indicated HIV drug resistance to be >15%.

The examination of plasma RNA for resistant HIV strains indicates circulating mutants, if any, including emergent polymorphisms. The proviral DNA testing reveals mutants that have emerged in individuals about a year back and have slowly replaced the native wild type derived provirus.

Two types of antiretroviral drug resistance assays that exist today are: Phenotypic assays and Genotypic assays. These assays can be carried out only in laboratories with recommended biosafety measures (minimum biosafety level 2) in place.

Phenotypic assays

The phenotypic assays amplify the RT and protease genes from the predominant quasi-species in the patient's plasma virus RNA or proviral DNA. These amplicons are then inserted into the laboratory virus lacking the genes. The hybrid virus is then propagated in cell cultures, and its ability to propagate in

the presence of varying concentrations of the drug is measured. Results are expressed as concentrations of drug required to inhibit 50% of growth (IC_{50}) in comparison to a wild type control strain.

Genotypic assays

The genotypic assays detect changes in the sequence of the relevant HIV1 gene. This measures resistance by detecting mutations in the HIV1 genome that leads to one or more specific amino acid substitutions in the HIV1 reverse transcriptase or protease enzymes. These specific changes cause drug resistance. Viral RNA from plasma or proviral DNA can be used for testing. The amplified products are then sequenced. The sequences are analysed using special software such as Stanford University Online software (http://hivdb.stanford.edu/), Los Alamos National Laboratories HIV Sequence Database (hiv-web.lanl.gov), http://www.hivresistanceweb.com/request/pda.shtml, etc.. Single genome amplification (SGA) may provide more accurate genotyping characteristics than conventional genotyping assay, where population-based sequencing is done. Importantly, the EQAS programme samples should be compatible to the locally circulating strains.

Table 4.1: Advantages and disadvantages of genotypic assays over phenotypic assays

Category	Genotypic assay	Phenotypic assay
Advantages	• Less expensive than phenotypic assays • Short turnaround time (<1 week) • May detect presence of resistance mutations before they have resulted in phenotypic resistance	• Interpretation similar to susceptibility testing of bacteria • Assesses the total effect of mutational changes • Good reproducibility • The threshold to define susceptibility is arbitrary
Disadvantages	• Interpretation requires knowledge of mutational changes • May show discrepancy with phenotypic assay	• Very expensive to set up and run • Turnaround time is more (3 weeks) than that of genotypic assays. • Slower to show resistance relative to genotypic assays

A third approach to resistance testing is the "virtual" phenotype. This assay is really a genotype resistance that is interpreted with the aid of a large database of samples with paired genotypic and phenotypic data. Viruses with genotypes that are similar to the patient's virus are identified by searching the database, and the average IC_{50} of these matching viruses is calculated. This information is then used to estimate the likely phenotype of the patient's virus. The major advantage of this approach is that it reduces complex genotypic data to simple phenotypic categories based on a rational, data-driven analysis of similar genotypes. The major disadvantage of this approach is that the confidence placed in the result depends on the number of matches, and on picking the right codons to incorporate into the database search. Correlation between actual and virtual phenotype will be weaker for newer drugs or in cases where there are fewer matches due to unusual genotypes. However, it needs more validation studies.

Monitoring of opportunistic infections in HIV disease

Morbidity and mortality in HIV disease is due to the occurrence of life-threatening opportunistic infections (OIs) during the natural course of the disease. These are the direct consequence of a decline in CD4 count. A wide variety of opportunistic infections (Table 5.1) are encountered in patients with AIDS which are caused by various microorganisms. Very often these represent reactivation of organisms that have been dormant in the host for several years. The incidence of these diseases increases as the patient's CD4 count declines. The pattern/repertoire of opportunistic infections may vary in different geographical areas. The knowledge of important OIs specific for particular areas/countries is useful for correct diagnosis and management of OIs.

Table 5.1: Common opportunistic infections in HIV/AIDS

Disease	Pathogen	Infection type
Bacterial		
Tuberculosis and non-tuberculous infections	*Mycobacterium tuberculosis* *Mycobacterium avium* complex	Pulmonary or meningeal or other extra-pulmonary or systemic
Salmonellosis	*Salmonella sp.*	Typhoid fever or diarrhoea
Bacterial pneumonia	*Streptococcus pneumoniae, Nocardia sp., Pseudomonas aeruginosa*	Pulmonary infection

Disease	Pathogen	Infection type
Fungal		
Candidiasis	*Candida albicans*	Oral thrush or vulvovaginitis
Cryptococcosis	*Cryptococcus neoformans*	Meningoencephalitis, or pulmonary or systemic disease
Aspergillosis	*Aspergillus sp.*	Pulmonary disease or sinusitis
Histoplasmosis	*Histoplasma capsulatum*	Pulmonary disease or disseminated
Penicilliosis	*Penicilium marneffei*	Pneumonitis or disseminated disease
Parasitic		
Pneumocystis jiroveci pneumonia	*Pneumocystis jiroveci*	Pneumonia
Toxoplasmosis	*Toxoplasma gondii*	Encephalitis
Cryptosporidiosis	*Cryptosporidium sp. (mainly C.hominis and C. parvum)*	Diarrhoea
Isosporosis	*Isospora belli*	Diarrhoea
Viral		
CMV infection	Cytomegalovirus (CMV)	Retinitis or encephalitis or esophagitis or colitis
Herpes	Herpes simplex virus (HSV), types 1 & 2	Recurrent oral or genital ulceration or bronchitis or pneumonitis or esophagitis
Herpes zoster	Human herpes virus type 3	Vesicular skin lesions
Warts and carcinoma *in situ*	Human papilloma virus (HPV)	Genital lesions and cervical carcinoma
PML	JC virus	Encephalitis

Utility of microbiological monitoring

Natural history studies from India show that persons with a CD4 T lymphocyte count of <200 cells/ul are 19 times more likely to die than those with a CD4 T lymphocytes count of >350 cells/ul Most of the diagnosed individuals have

more than one opportunistic infection. Patients who had more than one opportunistic infection were 2.6 times more likely to die than those who did not have an opportunistic infection.

Diagnosis of opportunistic infections

Infrastructure facilities should be established based on the level of the set-up required (Table 5.2). Although intermediate laboratories have sufficient facilities, further identifications and confirmations may be carried out by the central laboratory. The flow of specimens should be worked out from the lower to the higher level, and the flow of technical and scientific information and QA/QC procedures should be from the higher to the lower level.

Table 5.2: Suggested laboratory diagnostic procedures to be performed at different levels

Disease	Diagnosis at different levels		
	Peripheral	Intermediate	Central
Tuberculosis and non-tuberculous infections	AFB smear	AFB smear, culture	AFB smear, culture and sensitivity, PCR
Salmonellosis	–	Culture and sensitivity	Culture and sensitivity
Bacterial pneumonia	–	Culture and sensitivity	Culture and sensitivity
Candidiasis	Gram's staining	Gram's staining	Culture and sensitivity
Cryptococcosis	Negative staining, LA Test	Culture	Culture
Aspergillosis	10% KOH mount	Culture	Culture
Histoplasmosis	10% KOH mount	Culture	Culture
Penicilliosis	10% KOH mount	Culture	Culture
PCP	–	–	IFT
Toxoplasmosis	LA test	LA test	IFT
Cryptosporidiosis	–	Wet mount, Modified acid-fast staining	Modified acid-fast staining, IFT
Isosporosis	–	Wet mount, Modified acid-fast staining	Modified acid-fast staining, IFT

Disease	Diagnosis at different levels		
	Peripheral	Intermediate	Central
CMV infection	–	–	IFT, cell culture, PCR
Herpes	–	–	Cell culture, PCR, IFT
PML	–	–	PCR for JC virus
HPV	VIA	VIA/PAP smear	PAP smear/biopsy and PCR

IFT: immunoflourescent technique, LA: latex agglutination, VIA : visual inspection on application of acetic acid.

Role of the central laboratory in sharing data

The microbiological monitoring of opportunistic infections will require complementary activities of various partners in the laboratory network. The central laboratory (or National Reference Laboratories for the particular organism) shall act as the apex laboratory and collate data from other laboratories to feed them into the national programme. The central laboratory, in addition, shall train laboratory staff and assure appropriate management including safe collection and processing of biospecimens; act as country focal point for national coordination if there is a problem in identification and confirmation; act as technical resource centre; assure and/or assess quality in subordinate laboratories; prepare relevant guidelines and their distribution to other laboratories; maintain a repository of the isolates and reference strains and monitor the resistance in isolates against the specific antimicrobial agents.

5

Laboratory monitoring of side-effects of ART

Toxicity is related to the inability to tolerate the side-effects of medications and to significant organ dysfunction that may result on account of it. All the available antiretroviral agents have potential toxicities (Table 5.1). Careful monitoring of patients by laboratory investigations play a major role in better clinical management. Toxicity should be monitored clinically based on patient reports and physical examination, supplemented by a limited number of laboratory tests depending on the symptoms that arise and the specific combination regimen that is used.

Table 5.1: Adverse drug reactions of individual antiretroviral agents and laboratory tests

Agent	Affects quality of life	Serious, may require intervention	Serious and life-threatening	Laboratory tests
NsRTI (nucleoside reverse transcriptase inhibitor)				
Didanosine (DDI)	Diarrhoea, peripheral neuropathy	Pancreatitis, hepatotoxicity	Fatal pancreatitis with lactic acidosis	Liver function tests, amylase, lipase, lactate
Lamivudine (3TC)	Nausea, vomiting	Pancreatitis in children, increased anemia		Amylase, lipase, complete blood count
Stavudine (D4T)	Lipoatrophy, peripheral neuropathy	Hepatotoxicity, pancreatitis	Rapidly ascending neuromuscular weakness associated with lactic acidosis	Lactate, lipid profile, liver function tests, amylase, lipase
Zidovudine (AZT)	Headache; nausea, vomiting; myalgia	Neutropenia, megaloblastic anaemia lactic acidosis	Severe anaemia	Lactate, liver function tests, complete blood count

Agent	Affects quality of life	Serious, may require intervention	Serious and life-threatening	Laboratory tests
Emtricitabine	Nausea, vomiting, diarrhoea	Skin rashes, neuropathy, paresthesis	Lactic acidosis	Lactate
NtRTI (nucleotide reverse transcriptase inhibitor)				
Tenofovir (TDF)	Diarrhoea, headache, asthenia	Renal failure	Lactic acidosis	Renal failure tests
NNRTI (non-nucleoside reverse transcriptase inhibitor)				
Delavirdine (DLV)	Nausea, vomiting	Skin rash, hepatotoxicity		Liver function tests
Efavirenz (EFV)	Insomnia, somnolence, abnormal dreams, dizziness, decreased concentration, drowsiness	Depression, suicidal ideation, hallucination, psychosis, skin rash, hepatotoxicity, dyslipidaemia		Liver function tests, lipid profile
Nevirapine (NVP)		Skin rash, hepatotoxicity	Stevens-Johnson Syndrome, fulminant hepatic necrosis	Liver function tests
PI (protease inhibitor)				
Amprenavir (APV)	Nausea, vomiting, abdominal pain	Skin rash, dyslipidaemia,		Liver function tests, lipid profile
Indinavir (IDV)	Requires adequate daily fluid intake to prevent crystalluria; paronychia; hair thinningloss	Nephrolithiasis, renal failure, dyslipidaemia	Rarely haemolytic anaemia	Liver function tests, lipid profile, renal function tests, complete blood count
Lopinavir (LPV)	Diarrhoea, nausea, vomiting; perioral paresthesia, taste perversion	Dyslipidaemia		Liver function tests, lipid profile
Nelfinavir (NFV)	Diarrhoea	Diarrhoea may lead to severe dehydration, dyslipidaemia		Liver function tests, lipid profile
Ritonavir (RTV)	Nausea, vomiting; diarrhoea,abdominal pain, anorexia, perioral paresthesia, taste perversion	Dyslipidaemia		Liver function tests, lipid profile
Saquinavir (SQV)	Nausea, vomiting, abdominal pain	Dyslipidaemia		Liver function tests, lipid profile

Agent	Affects quality of life	Serious, may require intervention	Serious and life-threatening	Laboratory tests
Atazanavir (ATV)	Nausea, vomiting	Hyperbilirubinaemia nephrolithiasis, dyslipidaemia		Liver function tests, lipid profile
Fos-amprenavir (FPV)	Diarrhoea, vomiting, skin rashes	Dyslipidaemia		Liver function tests, lipid profile

Monitoring of adverse reactions

Monitoring toxicities of the drug can be done clinically based on patient reporting and physical examination. However, inclusion of limited laboratory investigations in the ARV monitoring will determine the severity of the toxicity and this will help physicians to change the dose and specific drug combination in the regimen. When the toxicity is related to an identifiable drug in the regimen, the offending drug can be replaced with another drug that does not have the same side-effect.

Monitoring of ART

The different levels of testing capabilities and types of specimen to be collected for monitoring of ART toxicity are described in Table 5.2.

Table 5.2: Suggested *testing* capabilities at different levels

Laboratory tests	Level 1: Peripheral	Level 2: Intermediate	Level 3: Central
Haemoglobin	Haemoglobinometer[1]	Haematology analyser[2]	Haematology analyser
Total and differential cell count	Microscopic - manual	Haematology analyser	Haematology analyser
Complete blood count	Microscopic - manual	Haematology analyser	Haematology analyser
Liver and renal function markers	–	Medium throughput autoanalyser[3]	High throughput autoanalyser[5]
Complete clinical chemistry markers, including serum electrolytes.	–	Medium throughput autoanalyser and ion selective electrode[4]	High throughput autoanalyser and ion-selective electrode
Pregnancy testing	Rapid test	Rapid test	Rapid test

Approximate cost of the instruments:100$[1], 10000$[2], 24000$[3], 40000$[4], 80000$[5]

- Patients with hepatitis B (HBV) and C (HCV) viruses should be monitored more closely for liver toxicity. HBV and HCV screening and testing may be undertaken in the particular community (eg. injecting drug users or IDUs) where prevalence is high.
- The critical values/panic values (abnormal values) of the laboratory results should be reported only after consultations with the treating physician.

Role of central laboratories

The laboratory-based monitoring of adverse drug reactions will require complementary activities of various partners in the laboratory network. The central laboratory (or National Reference Laboratories for the particular parameter) shall act as the apex laboratory and collate data from other laboratories to feed them into the national programme. The central laboratory, in addition, shall train laboratory staff and assure appropriate management including safe collection and processing of biospecimens; act as country focal point for national coordination; act as a technical resource centre; assure and/or assess quality in subordinate laboratories; prepare relevant guidelines and their distribution to other laboratories; help other laboratories by providing the information regarding the selection of reagent make and methods of the test; and establish and maintain normal ranges for haematology and chemistries.

6

Tuberculosis in HIV/AIDS

The WHO South-East Asia Region bears over a third of the global tuberculosis (TB) burden and ranks second after sub-Saharan Africa in the estimated number of people living with HIV/AIDS. Each year nearly three million cases of TB and 500 000 TB deaths are estimated to occur in the Region. Of the estimated six million adults living with HIV in the Region, about 60%-70% are likely to be infected with *Mycobacterium tuberculosis (M. tuberculosis)*. The extent to which HIV will contribute to the TB epidemic depends on the degree of overlap between the population groups infected with TB and those with HIV.

Pulmonary TB accounts for 60%-80% and extrapulmonary TB for 20%-40% of the total tuberculosis manifestations in HIV patients. Before the AIDS pandemic, non-tuberculosis mycobacteria (NTM) rarely caused serious illness, even in immuno-compromised individuals. The prolonged immuno-suppression of the cell-medicated immune system caused by HIV provides the opportunity for these relatively avirulent organisms to cause disease.

Impact of TB on HIV

TB is one of the most common causes of morbidity and mortality in people living with HIV/AIDS. TB accelerates the course of infection resulting in rapid progression from HIV infection to AIDS and shortens survival, even in those with high CD4 counts. *In vitro* studies have shown that TB increases HIV replication, immune activation and HIV viral load. The contribution of TB to AIDS deaths is substantial; up to 40% AIDS deaths are due to TB.

Drug-resistant TB

When a strain of *M. tuberculosis* becomes resistant to two or more "first-line" antibiotic drugs (i.e. isoniazid and rifampicin), it is called multidrug-resistant TB or MDR-TB. This can take up to two years to be treated, with drugs that are a hundredfold more expensive than those used in first-line treatment. When the bacteria becomes resistant to three or more "second-line" antibiotics as well (i.e. MDR-TB plus resistant to any of the fluoroquinolones and any one of the injectable second-line anti-TB drugs such as amikacin, kanamycin or capreomycin), it is classified as extremely drug-resistant TB or XDR-TB. Drug resistance usually arises when TB patients do not or cannot take their medicine as prescribed and drug-resistant mutations of the bacteria are allowed to replicate. People can also acquire MDR- and XDR-TB from others.

According to the *Fourth WHO Report on Drug Resistance in Tuberculosis (2008)*, MDR TB has been shown to be almost twice as common in TB patients living with HIV compared to TB patients without HIV. MDR-TB and XDR-TB are highly lethal in HIV-positive people, whose weakened immune systems render them unlikely to fight off the infection; studies show case fatality rates going over 90%. HIV-infected TB cases are more likely to be smear-negative, and delayed diagnosis of drug resistance has contributed to high death rates in people living with HIV.

Newer methods for rapid diagnosis of infection and drug susceptibility testing are now available to ease this problem. Acquired rifamycin resistance has been associated with HIV infection and anti-TB drug malabsorption has been documented in patient cohorts in settings of high HIV prevalence. Drug-resistant TB is, therefore, a major threat to the effectiveness of both TB treatment and anti-retroviral treatment programmes.

Laboratory diagnosis of TB

HIV co-infection with TB presents challenges to effective diagnosis of TB. The rapid rise of drug-resistant (DR) TB has further complicated TB diagnosis. However, only a small proportion of MDR-TB cases worldwide are being diagnosed and treated appropriately, mainly because of inadequate laboratory services.

Types of specimen

Sputum is the specimen of choice for diagnosis of pulmonary TB. Two specimens of sputum (one on-the-spot and one after an overnight collection) are to be examined over a period of two days. Specimens should be collected in sterile universal containers, and should have a fixed label for noting patient's details on the side. All forms of TB other than pulmonary TB are paucibacillary in nature. Depending upon the form of disease manifestation, several specimens such as sputum and/or gastric lavage , bronchoalveolar lavage (BAL), lymph nodes and other biopsy specimens, pus, ascitic fluid, and pleural and cerebrospinal fluid should be examined. If delay is anticipated, biopsy specimens may be collected in a suitable transport medium for sending them to the laboratory (to retain the viability of the organisms).

Techniques for detection of infection and drug susceptibility testing

The following are some of the commonly used laboratory tests for the diagnosis of TB and drug susceptibility testing (DST).

1. Sputum smear microscopy

Microscopy of sputum is of great value in the detection of open or infectious cases of TB, and is still the front-line tool for diagnosis of active TB. The establishment of a good sputum microscopy service is of prime importance in developing countries for the detection and treatment of open cases. Since the test can help determine if a person is infectious, it can also be used to monitor an active TB patient's response to treatment.

Smears are stained by the Ziehl-Neelsen (ZN) method, or by one of its various modifications. Grading of the positive smears gives a broad indication of the severity of the disease and the response to therapy. Although this is the simplest and cheapest laboratory test for the diagnosis of active TB, it has a few major shortcomings.

Limitations

- The sensitivity of the AFB smear test is known to be poor, varying between 30% and 70% depending on a number of factors relating to how and where the test is implemented. The sensitivity is further reduced in patients with HIV disease.

- As the test is based on sputum, it has particular difficulty in detecting extrapulmonary TB which occurs frequently in HIV-infected individuals.
- The test will also identify certain types of bacteria that are not *M. tuberculosis* (e.g. NTM, Nocardia, etc.) and so cannot always distinguish between TB and other infections.

Differentiation between M. tuberculosis and NTM

The identification of strains isolated as belonging to *M. tuberculosis* complex or NTM can be ascertained by performing a few simple tests, i.e. susceptibility to p-nitrobenzoic acid (PNB), niacin production test, catalase activity requirement and the morphological appearance. Important differences between *M. tuberculosis* and NTM are shown in Table 6.1.

Table 6.1: Differentiation between M. tuberculosis and NTM

Characteristic	M. tuberculosis	NTM
Growth rate	Slow grower	Slow/rapid grower
Temperature	37 °C	25–45 °C
Colony morphology	Dry, rough	Dry
Colony on solid media	Eugonic	Dysgonic
Colour of colony	Buff	Yellow, orange or creamy
Emulsification	Difficult	Easy
Cord formation	+	–
Niacin test	+	–
Nitrate reduction test	+	+/–
Growth on p-nitrobenzoic acid (500 ug/ml)	–	+

2. Sputum Culture

Demonstrating the presence of *M. tuberculosis* in the specimen by culture remains the gold standard for both diagnosis and drug sensitivity testing. However, sputum culture requires more sophisticated laboratory facilities with biosafety hoods and arrangements for safe disposal of culture media and sputum samples. The standard culture technique for mycobacteria is to decontaminate

by modified Petroff's method, where sputum is decontaminated with 4% NaOH and then inoculated on to Lowenstein-Jensen slopes. Alternatively, sputum specimens can be decontaminated and transported in 1% CPC (useful where specimens may take several days to reach the laboratory). The incubated cultures are examined once a week for eight consecutive weeks or until they become positive or contaminated. However, the usefulness of this technique is limited by several factors.

Limitations

- Conventional culture methods are very slow (Lowenstein-Jensen cultures take 20-56 days for diagnosis and 4-6 weeks after initial culture for DST; liquid cultures in 7H11 medium take 17-21 days for diagnosis and 3-6 weeks for DST).
- The sensitivity of culture is limited by the need to have at least 1000 bacilli/ml present in the sample to be cultured. HIV positive patients and children have difficulty in producing sputum, and sputum culture will not detect extrapulmonary forms of TB frequently found in HIV-positive patients.
- Culture-based tests are difficult to implement in the field. They require dedicated facilities and staff, with specific requirements for training, quality assurance, biosafety and equipment.

3. Culture-based rapid methods for detection of M. tuberculosis

Some more rapid culture methods have been developed (e.g. BACTEC-MGIT) based on liquid culture media that are commercially available, though most of them are difficult to implement in the field due to the complexity of the technique or the required equipment. There are also some emerging simplified culture techniques (e.g. MODS) that can reduce time to diagnosis or DST that seem more appropriate for use in resource-limited settings.

(a) Rapid liquid TB culture medium: BACTEC MGIT 960 Automated (Becton Dickinson, US)

The MGIT system is based on a plastic tube containing 7H9 broth together with a fluorescence quenching-based oxygen sensor. When inoculated with *M. tuberculosis*, consumption of oxygen produces fluorescence when illuminated by a UV lamp. The fully automated version can incubate up to 960 samples for *M. tuberculosis* diagnosis and DST for first-line drugs. The advantage of

this technique is the increased speed of diagnosis (7 days for sputum positive, up to 42 days for a negative result). DST results can be obtained in 8-12 days (starting from culture). On the other hand, the technique has the following disadvantages:

Limitations

- MGIT can only be used in a biosafety level 2 (BSL-2) culture facility, which limits its implementation.
- Liquid media are technically limited as they are prone to contamination.
- The machine is extremely expensive and requires stringent maintenance. Further, the company is the only source for the media.
- Technical personnel need to be specifically trained.

The manual system is cheaper than the automated system. Here detection is performed by the technician visually using a hand-held UV lamp. The MGIT machine is not required.

(b) Microscopic observation of drug susceptibility (MODS) assay

This method is based on direct inoculation of the selective 7H9 liquid culture medium in 24-well plates with sputum specimens subjected to digestion and decontamination. Detection of the typical cord formation (microcolonies) of *M. tuberculosis* in the wells on microscopic examination constitutes the basis of diagnosis. Growth or the lack thereof in drug-containing wells as compared with growth in drug-free wells is the basis for reporting the results as "susceptible" or "resistant". The MODS assay is rapid, low-cost and highly accurate for the detection of TB and MDR-TB. However, it has the following disadvantages.

Limitations

- The methodology is delicate and requires very experienced personnel.
- As the test is performed in liquid medium and needs to be handled often, it is more of a biosafety risk for laboratory staff.
- The test requires a BSL-2 environment and an inverted microscope which are seldom available in field laboratories.

4. Blood-based methods for detection of tuberculosis infection

(a) QuantiFERON-TB Gold Test

The QuantiFERON-TB Gold T est (QFT-G, Cellestis Limited, Australia) was the first blood test to be approved for the diagnosis of latent tuberculosis infection (LTBI). This test detects the release of interferon-gamma (IFN- γ) in fresh heparinized whole blood from sensitized persons when it is incubated with mixtures of synthetic peptides representing two proteins present in *M. tuberculosis*: early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). These antigens impart greater specificity than is possible with tests using purified protein derivative as the TB antigen.

Limitations

- QFT-G is not a confirmatory test; it is approved as an aid for diagnosing both LTBI and TB disease. As with the tuberculin skin test, if results of this test are indicative of TB disease, additional diagnostic evaluations (viz., chest radiography, bacteriological studies, serology for HIV, etc.) should be performed before or at the same time as the QFT-G and should not be delayed while awaiting QFT-G results.
- False negative tests are as common as with the PPD skin test, and studies evaluating the sensitivity and specificity of QFT-G in HIV-infected populations are underway.

(b) T-SPOT.TB Test

T-SPOT.TB test (Oxford Immunotec Ltd, UK) is a simplified ELISPOT test that detects effector T-cells that respond to stimulation by *M. tuberculosis* antigens by producing IFN-γ. This gives an overall measurement of the antigenic load in the immune system, and can reveal the presence of even sub-clinical disease. Since it does not rely on the production of a reliable antibody response or recoverable pathogen, the technique can be used to detect conditions such as latent TB. T-SPOT.TB results are available the next day and the test claims a sensitivity of 96%.

Limitations

- This test is again intended for use as an aid in the diagnosis of TB infection and should be interpreted only in the context of the overall clinical picture, X-ray, risk to contract TV, previous infections, and other medical/diagnostic tests.

5. Molecular method for detection of M. tuberculosis

Line probe assay (LiPA)

INNO LiPA (Innogenetics, Belgium) is a reliable, simple, rapid and informative tool for the early detection and characterization of mutations associated with rifampicin resistance in *M. tuberculosis* in the clinical laboratory setting, and may constitute an important molecular method for the control of TB.

The assay is based on the principle of reverse hybridization. Specific oligonucleotides are immobilized at known locations on membrane strips and are hybridized under strictly controlled condition with biotin labelled PCR products of the rpoB gene of *M. tuberculosis*. If a mutation is present in any of the PCR products, the corresponding probe will be prevented from hybridizing under the stringent hybridization and washing conditions used. Consequently, the absence of hybridization signal with one of the probes would be indicative of the presence of mutation.

Limitations

- The utility of the assay is restricted to the identification of a limited number of mutations only.

Though the assay can identify mutations in the rpoB gene, it does not give precise information about the nature of the mutation; this requires further testing.

7

New technologies in HIV diagnosis and ART monitoring

High costs of assays used in diagnosis and monitoring and resource limitations are the major concerns for HIV control and implementing treatment programmes. Newer technologies providing economical assays are very important for such programmes. The specific areas where such assays will be needed are:

- HIV diagnosis
- CD4 T lymphocytes enumeration
- Plasma viral load determination

The new technologies must be properly validated before applying them in resource poor settings and should follow a strict quality control regimen in the programme.

HIV diagnosis

Conventional HIV diagnostic methods have disadvantages because of the need for cold chain for sample transport and storage as well as for conducting invasive procedure. Some of the newer approaches obviate these shortcomings.

Tests on samples other than blood (saliva and urine): Commercial tests are available for the detection of anti-HIV antibodies in urine (Calypte Biomedical Corp, USA; Epitpope Inc, USA) and saliva specimens (OraSure

Technologies, USA and BioMerieux Inc, France; Calypte Biomedical Corp, USA) have shown promising results. The tests that use saliva samples are non-invasive and may be performed in peripheral centres but are more expensive than blood tests.

Tests using dried blood spot: Antibody detection on dried blood spots (DBS) has been employed in field surveys in infections other than HIV. The advantages include stability at room temperature obviating the need for cold chain, suitability for community-based surveys, and adaptability for collection of a large number of samples. It can be adopted for confirmation in Western Blot. However, no data on borderline samples/recently infected individuals with probable low titer antibody are available. The system has still to undergo vigorous quality assessment. Some of the commercially available kits for antibody detection may not be suitable for this strategy.

Newer technological approach in CD4 T lymphocytes enumeration

The newer assays are being developed to provide immediate CD4 T lymphocytes readings for the point of care. The attempts are being made to design such technologies for the rapid assay to provide accurate results.

Microchip-based systems

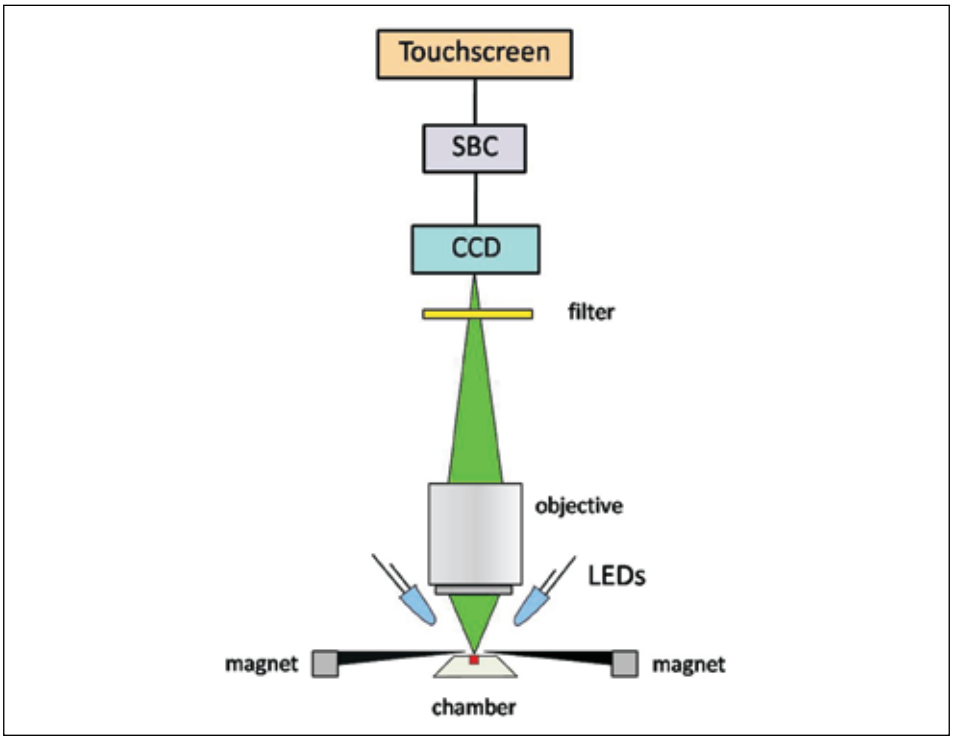
A new methodology using a microchip-based detection system, called an electronic test chip (ETC), has been developed recently. The ETC is a promising technology with wide applications and can detect chemicals and proteins in solution. Each chip contains microspheres in a small chamber through which whole 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 laboratory for viewing and comments.

- ***Microchip technologies (Image cytometer)***

This SP image cytometer was developed by the Biophysical Engineering Group, Faculty of Science and Technology, University of Twentee, Enschede, The Netherlands. The instrument is an automatic image cytometer that takes a fluorescent image of immuno-fluorescently labelled CD4 T lymphocyte that have been magnetically collected at the upper-surface of a sample chamber inserted between the poles of a magnet. Light emitting diodes (LED) are used as excitation light sources. A standard microscope objective collects the fluorescence emitted by cells and captured after filtering on a single CCD camera. The acquired image is then transferred to a single board computer (SBC) that is operated by a touch-screen monitor, and analysed using a dedicated image analysis algorithm to determine the number of CD4 T lymphocyte per ul of whole blood.

The system is battery-operated and robust. No highly qualified personnel are needed for the preparation of samples and the handling of the instrument. It is a technically simple and less expensive (costing only US$ 3000) alternative

Figure 8.1: **Image cytometer: Principle**



to the conventional flow cytometer, thus making this system more appropriate for use within laboratories with limited infrastructure, for example, in peripheral laboratories.

There are some new approaches using the existing flow cytometry instruments. For example, the new software and new reagent for the BD FACSCount instrument; one software gives both absolute CD4 and CD4 percentage.

New reagent and software for the BD-FACSCount:

New CD4 reagents and software from Becton Dickinson on the FACSCount system can provide absolute CD4 T lymphocytes and percentage results (single-platform) in an easy-to-use single-tube assay. The software identifies the lymphocyte populations of interest and calculates absolute CD4 T lymphocytes counts (cells/u l) and CD4 T lymphocytes percentages automatically, thereby saving time, minimizing human error and reducing costs.

Viral load measurement

Measurement of plasma virus load is currently being used for monitoring the progression of HIV infection as well as to monitor the efficacy of antiretroviral treatment. Current methods of determining plasma virus load include quantitative RT-PCR, branched DNA technology and NASBA. These technologies are expensive and may not be affordable for programmes in developing countries. A few alternative approaches that are under investigation are mentioned below:

- *Real time PCR:* The technique can be used as a modified cost-effective approach for viral load measurement with around 30% to 50% cost reduction. The recent validation studies from different countries have shown positive results. Few commercial kits (eg. ABBOTT RT PCR and Roche Tagman RT PCR) are available for real-time PCR application for determining plasma virus load.

Plasma preparation tube (PPT) for viral load measurement: PPT from Becton Dickinson has been validated for the transportation of plasma samples from remote sites to the testing laboratory and has shown good results. This tube provides a closed collection, preparation and transport of plasma sample. The major advantage of this tube is that the separation of plasma is done

without opening the tube on-site and the tube can be transported to the testing laboratory in an ice-pack (dry ice is not required). This collection system is very useful, particularly for places which do not have facilities to separate plasma specimens or ship frozen plasma on dry ice.

- *Viral load measurement by flow cytometry:* The quantifications of PCR products by dedicated flow cytometry is of significance and is under development (Partec, Germany).
- *Viral load measurement on dried blood spot (DBS)/plasma dried spot (DPS):* DBS and DPS have been evaluated against the gold standard for viral load measurements. However, continuing research on DBS is needed to better understand the effects of variables such as drying time, humidity and temperature on RNA stability.

8

Laboratory infrastructure and networking

The organization of laboratories in any country is usually a three- or four-tier system with various possible functional linkages between them. There must also be transport and communication facilities between peripheral and intermediate laboratories for referral of samples and patients, procurement of supplies and personal discussion. Laboratory investigations can be established based on the level of the laboratory set-up required for that region, as follows:

Level 1: Peripheral laboratories

Functions: These laboratories provide technical support for the diagnosis and clinical management of HIV-infected individuals (monitoring of HIV and ART). This level of laboratory will perform some simple testing, such as HIV rapid testing, haemoglobin estimation, TB smear examination, cell count (TC and DC), pregnancy testing and basic biochemistry tests which do not require enhanced skills. The laboratory should participate in external proficiency assessment for the tests being conducted in the laboratory

Staffing*:* The staff in peripheral laboratories should include preferably at least one microbiologist/pathologist (with a Master's degree in pathology/ medical microbiology), in addition to one laboratory technician and one laboratory assistant/attendant.

Space**:** Space available in peripheral laboratories should include at least one laboratory-cum-office/record room (approximately 5 metres x 3 metres) and one store-room which can be used for other services also (approximately 5 metres x 3 metres).

***Equipment*:** Light microscope (binocular) – 1; small refrigerator (165-litre capacity) – 1 Bio-safety centrifuge (maximum RPM 5000) – 1; autoclave – 2; safety spectacles; micropipettes (variable volume 40–200 ul) – 2; micropipettes (variable volume 5–40 ul) – 2; Bunsen burner with LPG; sample racks; computer with printer – 1; needle destroyer – 1.

***Support facilities*:** Other facilities include prompt laboratory supplies (glass slide, cover slip, staining solutions, microtips, transport media, sterile swabs, vacutainer or syringe, test tubes, sample containers, serological pipettes, Pasteur pipettes, diagnostic kits, etc.), work bench (at least 0.5 x 6 metres in size and made of concrete), chairs and cupboards, supply of safe water, stabilized power supply (power disruptions should not be for a duration longer than two hours), telephone and Internet connection, waste disposal facilities, PEP availability, separate sinks for biowaste and clean work in each laboratory area, eye-wash stations, fire extinguishers, etc.

Level 2: Intermediate laboratories

***Functions*:** The functions expected to be performed by the intermediate laboratories include support to clinical diagnosis/public health, quality assurance, logistic and technical support and training of staff for peripheral laboratories. These laboratories also serve as links between peripheral laboratories and the state/central laboratory for the following: collection, storage and analysis of data; distribution of reagents; media; preparation of laboratory manuals and standard operating procedures for the functions to be carried out at peripheral as well as intermediate laboratories; purchase of equipment; supervision of peripheral laboratories; conduct of EQA for peripheral laboratories; to take part in EQA organized by state/central laboratories; and to send samples to higher/reference laboratories for characterization of isolate/confirmation of diagnosis.

***Staffing*:** One qualified laboratory supervisor/manager (with a Master's degree in pathology/medical microbiology), three Technical Assistants (undergraduates in biochemistry or microbiology plus Diploma in Medical Laboratory Technology, three Technicians (cum-Phlebotomist), one Laboratory Attendant-cum-Housekeeper and one Clerk-cum-Storekeeper.

***Space*:** Space available in level-2 laboratories should include one room (approximately 5 x 5 metres) for microbiology, one room for sterilization, media preparation (approximately 3 metres x 3 metres), one room (approximately 5 x 5 metres) for serology and biochemistry, one room (approximately 5 x 5

metres) for haematology and immunology, store-room (approximately 3 metres x 3 metres), office (approximately 3 metres x 5 metres) and one room for staff resting/dining (approx 3 metres x 3 metres).

***Equipment*:** Light microscope (binocular) – 2(one with fluorescence and phase contrast attachment); biosafety cabinet (class II) – 2; autoclave – 2; incubator – 2; hot air oven – 1; water bath – 2; VDRL shaker – 1; autoanalyser – 1; and at least one semi-autoanalyser as back-up; computer with printer – 3; refrigerators (>200 litre capacity) – 3; biosafety centrifuge (the maximum RPM 5000) – 2; weighing balance – 1; pH meter – 1; ELISA washer –1; ELISA reader – 1; flow cytometer (single or dual platform) – 1; Western blot rocker – 1; haematolology analyser (3 part) – 1; deep freezer (-20 °C or colder) – 1; inspissator – 1; distilled water apparatus – 1; safety spectacles/protective glass shields; micropipettes (variable volume 200 ul–1000 ul); micropipettes (variable volume 40–200 ul); micropipettes (variable volume 5–40 ul); needle destroyer; Bunsen burners with LPG; and sample racks.

***Support facilities*:** Other facilities include prompt laboratory supplies including plastic wares, glassware and diagnostic kits; work bench (at least 0.5 x 6 metres in size of concrete with granite table-top) in each laboratory area; air-conditioned laboratory rooms; sufficient chairs and cupboards; continuous supply of quality water; stabilized power supply with battery back-up for at least four hours (power cuts should not be of more than 2 hours' duration); telephone and Internet connection; waste disposal facilities; PEP availability; readily available technical supports for all the equipment; separate sinks for biowaste and clean work in each laboratory area; eye-wash stations; fire-extinguishers, etc.

Level 3: Central or national laboratories

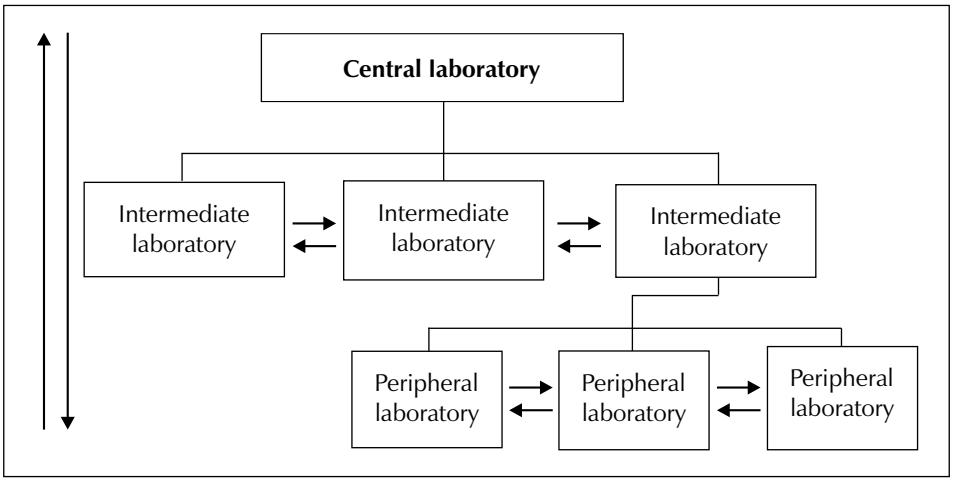
***Functions*:** The national laboratory shall provide technical support to intermediate and peripheral laboratories by training, maintenance of laboratory QA/QC, purchase of equipment, validation and selection of diagnostic kits, preparation of testing algorithms, supervision, preparation of laboratory manuals and standard operating procedures for the functions to be carried out at all three levels of laboratories, dissemination of newsletter, and support to participate in national/international EQAS programme and to conduct EQAS for intermediate and peripheral laboratories. The laboratory can also characterize isolates and confirm the diagnosis.

These laboratories should have more facilities than those for the intermediate level, plus additional facilities such as cell culture facility, facilities to carry out techniques like PCR, viral load estimation. The laboratory should also have a flow cytometer to run a QA programme for intermediate laboratory where an alternate system for CD4 count estimation might be in use. This level of laboratories should be conducting continuing education, workshops and hands-on training programmes.

The functions of the laboratories at various levels have been described in Chapter 1.

Health services are utilizing laboratories more extensively now than ever before. Laboratories play an important role in the diagnosis of HIV infection as well as monitoring of antiretroviral treatment. The demand for quality results from laboratories has been echoed by all the health-care professionals caring for HIV-positive individuals. Unreliable laboratory results are known to have serious consequences for the health of the individual as well as the community. It is important to have a system of networking, peripheral with regional and central laboratories of a country, in order to facilitate quick and accurate processing and testing of specimens collected from patients, quick dissemination of information to the peripheral levels, as well as to run a quality assurance programme. One possible way of networking of laboratories is shown in Figure 8.1.

Figure 8.1: **Suggested networking of laboratories**



9

Quality system in the laboratory

A quality laboratory report helps the physician to establish proper diagnosis rapidly and supports better health care for the patient. Apart from providing accurate results, maintaining quality in laboratory services also helps in generating confidence among patients in the health care system, creating a good reputation for the laboratory, reducing costs by avoiding repetition of tests, sustains motivation in laboratory staff, helps in the accreditation of the laboratory and prevents legal suits and associated complications. The international standard requirement for the implementation of quality standards for the medical laboratories is described in ISO15189:2007 document.

Quality system

Quality system is a part of overall quality management that aims to ensure consistency, reproducibility, traceability and efficacy of products or services. It is defined as the organizational structure and resources needed to implement quality requirements.

A well-defined quality system is a must for ensuring quality. It is a part of overall quality management which aims at ensuring the consistency, reproducibility, traceability and reliability of the products or services. A quality system is defined as the organizational structure and resources needed to implement quality requirements. The International Organization for Standardization (ISO) defines a quality system as the organizational structure, responsibilities, procedures and resources needed for implementing quality management.

A quality system has the following five key elements:

- Organizational management and structure
- Quality standards
- Documentation
- Training
- Assessment

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 staff members of the organization.

Quality standards

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

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. Documents may be stored either as hard copy or electronically. The standard operating procedure (SOP) is a clear and precise documented description of any activity including sequence of operation, method to be used, equipment to be used and records to be kept.

Training

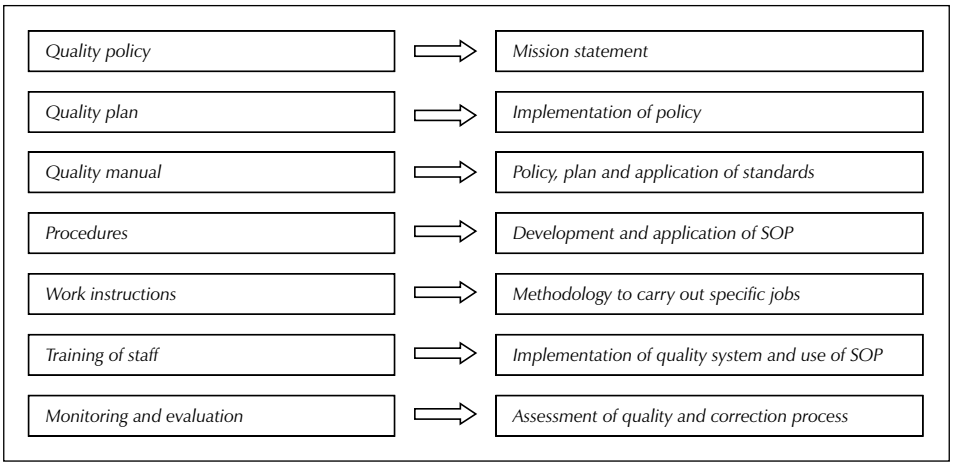
The quality system is only as good as the staff who actually works with it. No matter how good the quality system is on paper, if theory cannot be translated into practice, quality cannot be achieved. Training must also include an understanding of why quality is important. Training should be competency-based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

Assessment of quality system

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 (EQAS) are other tools, the results of which should guide the management in further improving quality.

Development of a quality system

The development of a quality system can be done in a stepwise approach as shown in the figure below:



Key definitions used in quality system

Internal quality control (IQC)

Internal quality control is a set of procedures undertaken by the staff of the laboratory to ensure quality from the collection of specimens, the performance of the test upto analytical results, and the procedure being planned, ordered and followed up by the staff itself.

Sensitivity

Diagnostic sensitivity: The incidence of true positive results obtained when a test is used for patients known to have disease or condition.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \text{ X } 100$$

Analytical sensitivity: The ability to detect small differences in concentration in a series of specimens.

Specificity

A measure of the ability of a test to indicate the absence of a component in a specimen when it is truly negative for that component, or a measure of the ability of a test to measure accurately one component in a specimen without interference by other components.

(1) A measure of an ability of a test to give a negative result in the absence of disease.

(3) $$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \text{ X } 100$$

Efficiency

The ability of a test to give a positive result on positives and negative results on negatives.

$$\text{Efficiency} = \frac{\text{True positive} + \text{True negative}}{\text{True positive} + \text{False positive} + \text{True negative} + \text{False negative}} \text{ X } 100$$

Validation

Confirmation by examination and provision of evidence that specific requirements for specific intended use are met.

More information on the quality system is available in the *WHO-SEARO Guidelines for Establishment of Accreditation of Health Laboratories*, HLM-394, 2007.

10

Bio-safety practices, accidental exposures and post-exposure prophylaxis

Safety procedures include laboratory protection of the material to be tested, environment and the staff. Personal and laboratory safety can be achieved only by informed, trained responsible individuals through the application of standard precautions.

Standard precautions are simple infection control measures that reduce the risk of transmission of pathogens through exposure to blood or body fluids. The laboratory personnel should be trained in safe handling of the clinical specimen and proper disposal of biowaste materials. The detailed SOP for biosafety must be available in the laboratory.

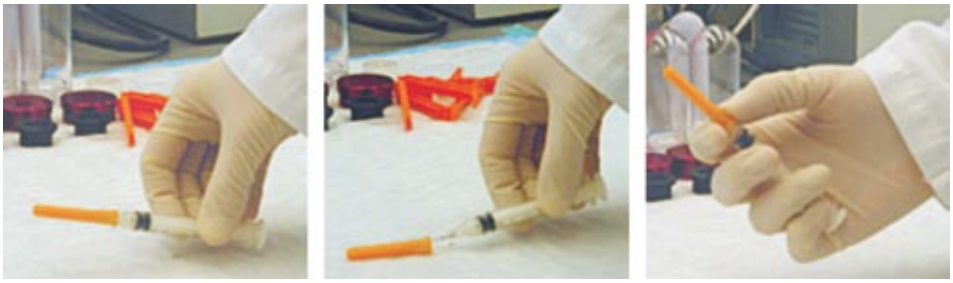
Table 10.1: Biosafety practices in the laboratory—Dos and Don'ts.

Dos	Don'ts
• The laboratory should be kept neat, clean and free of materials that are not pertinent to the work. • Training in safety measures should be an integral part of new employee's induction (including support staff) and that must be documented. • Use barrier protection with gloves, gowns/aprons, face shield, goggles whenever splashes of infectious fluid are expected.	• Do not do the phlebotomy without gloves. • Do not allow personnel to handle laboratory work until he/she completes the full course of hepatitis B vaccine. • Do not recap, bend or break used needles – but if recapping has to be done, single-handed method may be used (Figure 10.1). • Never pass used sharps directly from one person to another.

Dos	Don'ts
• Use biosafety centrifuges for sample processing. • Use puncture-proof containers for disposing of sharps and these must be as close to the point of use as possible. • Carefully place used plastic syringes, needles and sharps in the containers, then disinfect by chemical or physical methods, i.e. boiling or autoclaving, before disposing into incinerator. • Keep all sharps and sharp disposal containers out of easy reach. • Cuts in hand should be properly covered with waterproof occlusive bandages. • Biosafety cabinets must be used whenever handling biospecimens, which is likely to produce aerosols or infectious droplets. • Traffic behind the working personnel should be avoided. • Disinfect (with 0.5%–1% sodium hypochlorite solution) workplaces when procedures are completed or after any spillage of blood or other potentially infectious material. • Label the biowaste materials container with the "Biohazard label" • Use biosafety procedures for disposal of laboratory waste.	• Laboratory refrigerators should not be used for storing drinking water or food; refrigerators should be labeled with biohazard symbol. • Open-toed footwear should not be worn in laboratory. • Mouth pipetting must be strictly forbidden. • Do not use the sharp disposal container when it is three quarters full. • Do not forget to wash (with soap) hands after handling infectious materials and before leaving the laboratory working areas. • Do not touch blood spills with your bare hands. • • Do not pass on the sharps to other personnel to dispose; dispose of sharps yourself immediately. • Never eat, drink or apply cosmetics in a laboratory. • No untreated bio-waste should be stored beyond a period of 48 hours.

- **Management of spills:** If there is a spillage of blood or other body fluid, cover with a 10% sodium hypochlorite solution, leave it for at least 30 minutes, cover with gauze, cotton or sponge, and finally wipe away with gloved hands. If spills involve any broken glassware, it must be picked up using mechanical means such as a brush and dustpan, or forceps. In cases where the absorbent becomes saturated with blood and bleach, the spill clean-up materials should be autoclaved prior to being disposed in normal trash.

Figure 10.1: **Single-hand method of recapping needle**



- **Biowaste management:** Waste is defined as any solid, liquid or gaseous material that is no longer used and will either be recycled, disposed of or stored in anticipation of treatment and/or disposal. All laboratories should develop a comprehensive waste management programme that ensures the safe handling and disposal of all laboratory wastes. The programme must be tailored to meet the specific needs of the individual laboratory and should incorporate all applicable legal requirements. The basic goals of this programme are:
- to operate the laboratory in compliance with all applicable legal requirements and good laboratory practices, and
- to manage the wastes generated in a manner that protects laboratory workers, the environment and the community.
- Biowaste should not be mixed with other wastes and the biowaste should be segregated into containers/bags at the point of generation itself. The three important steps in biowaste management are:

  (1) segregation of waste

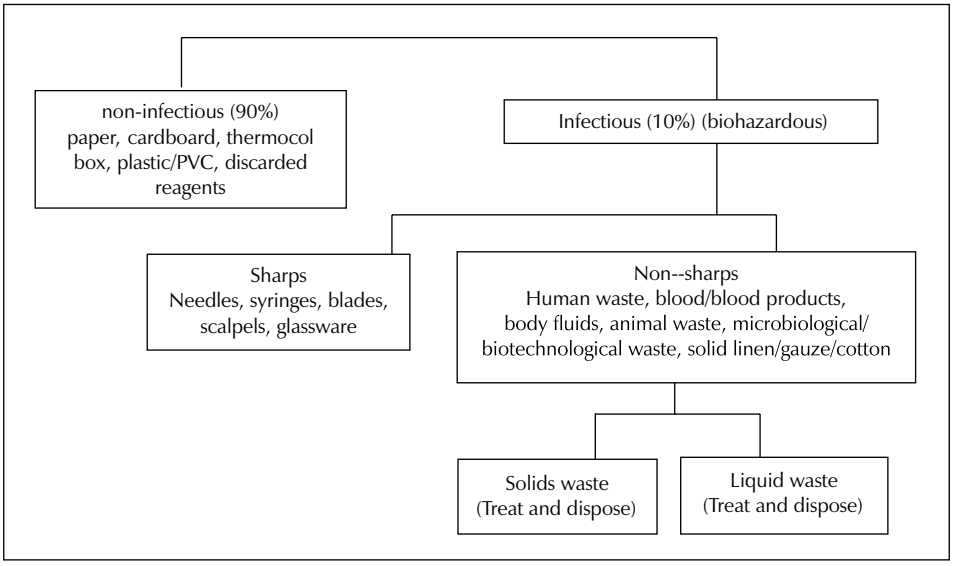
  (2) disinfection

  (3) appropriate disposal
- Colour-coded containers marked with the universal biohazard symbol (figure 10.2) should be used. Plastic liners should be used inside the biowaste containers.

Figure 10.2: Biohazard label

- The biowaste generated in the laboratory should be segregated (Figure 10.3) and before disposing, the waste should be pre-treated. The disposal should be done strictly in accordance with the options provided in Table 10.2. Package all waste to prevent spills, leaks or breaks during transportation.

Figure 10.3: **Segregation of laboratory waste**



- **Management of solid waste:** Solid biohazardous waste must be pre-treated by decontamination (such as autoclaving) before disposal. All waste must be collected in biohazard bags or closed, leak-proof, labelled containers, to prevent spillage or protrusion of contents during handling or transport.
- **Management of liquid waste:** Liquid waste such as human blood and blood components such as serum, plasma, etc., and human body fluids should be considered as potentially infectious. Before disposing, this liquid waste should be decontaminated by adding a sufficient volume of sodium hypochlorite to obtain a final concentration of at least 1%. A 30-minute exposure to 1% sodium hypochlorite may be sufficient to ensure effective decontamination. Commercial liquid household bleach typically contains a concentration of 5.25% sodium hypochlorite. A 1:5 dilution of household bleach will produce a 1% sodium hypochlorite solution. After decontamination, the waste could be disposed in the sewer.
- **Management of sharps:**
  - Use needle destroyer if available. If not discard into puncture-proof container with 1% hypochlorite and keep for minimum 30 minutes.
  - Carefully place used plastic syringes, needles and sharps in the containers, then disinfect by chemical (1% hypochlorite) or physical methods, i.e. boiling for 20 minutes or autoclaving at 121 ^{o}C at 15 PSI for 15 minutes before disposing into incinerator.
  - Any broken glassware must be picked up using a mechanical means, such as a brush and dustpan, or forceps. In cases where the absorbent becomes saturated with blood and bleach, the spill clean-up materials should be autoclaved prior to being disposed in the normal trash.

Table 10.2: Colour coding and type of container for disposal of biowaste

Biowaste category	Biowaste class	Type of container	Colour coding	Treatment/disposal option
No. 1	Human anatomical waste, blood and body fluids	Single-use containers/ Plastic holding bags	Red	Incineration/deep burial
No. 2	Animal and slaughter house waste	Single-use containers/ plastic holding bags/sacs	Orange	Disinfection & deep burial
No. 3	Microbiology & biotechnology waste	Single-use containers/ plastic holding bags	Yellow	Autoclaving / microwaving & incineration
No. 4	Waste sharps	Reusable/single-use sturdy containers of plastic, glass or metal	Blue	Shredding & deep burial
No. 5	Discarded medicines	Re-usable/sturdy cardboard/glass/plastic holding bags	Blue	Shredding & deep burial
No. 6	Soiled wastes (linen)	Plastic bags/sacs	Yellow/ black	Disinfection and machine cleaning
No. 7	Disposables (other than sharps)	Reusable/sturdy containers/plastic holding bags	Yellow/ black	Disinfection-chemical/autoclaving, shredding, burial

Occupational exposure to blood/other body fluids and post-exposure prophylaxis (PEP)

Health-care workers and laboratory staff are normally at very low risk (Annex 3) of acquiring HIV infection during management of HIV-infected patients. Appropriate post-exposure management guidelines form an important element of workplace safety. The technical personnel should be familiar with the safety precautionary measures. Avoiding occupational blood exposures is the primary way to prevent transmission of HIV, HBV and HCV in health-care settings. However, hepatitis B immunization and appropriate post-exposure management are integral components of a complete programme to prevent infection following bloodborne exposure. The average risk of acquiring HIV infection after different types of occupational exposure is low compared to risk of infection with HBV or HCV. In terms of occupational exposure the important routes are needle stick exposure (0.3% risk for HIV, 9–30% for HBV and 1–10% for HCV) and mucous membrane exposure (0.1% for HIV). The adherence to universal precautions at the workplace would reduce occupational exposure substantially.

Post-exposure prophylaxis (PEP) refers to the comprehensive management (Tables 10.3 and 10.4) given to minimize the risk of infection following potential exposure to bloodborne pathogens (HIV, HBV or HCV). This includes pre-test counselling, risk assessment, relevant laboratory investigations based on information gathered from the HCW and the source, first aid and, depending on the risk assessment, the provision of short-term (four weeks) of antiretroviral drugs, with follow-up and support. In order to ensure that an exposed person has access to prophylactic therapy in a timely manner, it is recommended that PEP drugs be kept available round-the-clock and health-care workers should know through in-house training where to get PEP as and when required.

The decision about whether or not to offer PEP should be based purely on clinical considerations of risk. The provision of information regarding PEP should be confidential, including information about HIV testing, PEP provision and the reasons for seeking PEP. The informed consent needs to be obtained for the administration of PEP. The PEP must be initiated as soon as possible after the exposure, preferably within two hours and not later than 72 hours; PEP is believed to be most effective if initiated within 48 hours of exposure.

The risk of infection varies with:

(1) type of exposure (superficial or deep injury);

(2) the amount of blood involved in the exposure;

(3) amount of virus in patient's blood at the time of exposure (patient's viral load); and,

(4) Whether PEP was taken within the recommended time (not later than **72 hours** after exposure).

What to do after a needle stick injury?

(1) Wash the injured site thoroughly with soap and water (antiseptics may be used).

(2) If as a result of a laboratory accident the skin is broken the wound should be cleaned and irrigated with a mild disinfectant such as chlorhexidine with cetrimide

(3) Administer post-exposure prophylaxis (PEP) for HIV, based on institutional policy after evaluation of risk.

Mucosal exposure

If there is an accidental exposure of the blood or other body fluids to mucosal surfaces (eg. mouth, nose or eyes) flush the exposed area with a large amount of water. The splashes into the eye should be flushed using an eye wash fountain for 15-20 minutes. PEP should be initiated as soon as possible.

Table 10.3: Recommended procedures for post-exposure management

Infectious agent	Post-exposure prophylaxis
HIV	Antiretroviral therapy (2-drugs or 3-drugs regimen)
HBV	Hepatitis B immune globulin (HBIG) and/or hepatitis B vaccine
HCV	No vaccine or chemoprophylaxis available

Table 10.4: Clinical management of HIV PEP: a summary

Procedure	Recommended action and notes
Eligibility for PEP	Exposure within 72 hours Exposed individual not known to be infected with HIV Significant exposure Person who was the source of exposure is HIV infected or has unknown HIV status and has not had any exposure within the prior three months
Informed consent for PEP	Information about risks and benefits Consent may be given verbally
Medicine	The standard PEP regimen should comprise two NRTIs (usually part of first-line antiretroviral therapy medicines). Three-drug regimens, comprising two NRTIs plus a boosted protease inhibitor may also be used depending on risk.
Duration of therapy	28 days
HIV testing with informed consent and pre-and post-test counselling according to protocols	Baseline HIV test in exposed person Follow-up HIV testing 3-6 months after exposure Rapid HIV test of the source person if feasible and based on informed consent

Procedure	Recommended action and notes
Additional laboratory evaluations	Pregnancy testing Haemoglobin (for zidovudine-containing PEP regimens) Hepatitis B and C screening if available and based on the prevalence of the diseases
Counselling	For adherence, side effects; risk reduction; trauma or mental health problems; and social support and safety
Referral	Referrals as appropriate
Record-keeping	Maintain accurate, confidential records
Follow-up – clinical	Assess and manage side-effects Assess and support adherence

Drugs recommended for HIV post-exposure prophylaxis

The recommended duration of PEP for HIV infection is 28 days. The first dose of it should always be offered as soon as possible after exposure. Once commenced, the full PEP should be taken unless there are specific reasons to stop (e.g. source is tested and found to be HIV negative). A two-drug regimen (basic regimens) is preferred given the relative toxicity of antiretroviral medicines, the side effects and their negative impact on adherence to PEP prophylactic treatment. Moreover, two-drug therapies are likely to be sufficiently potent to prevent HIV transmission.

The preferred 2-drug regimen is zidovudine (300 mg twice daily) + lamivudine (150 mg twice daily) and an alternative regimen is stavudine (30 mg twice daily) + lamivudine (150 mg twice daily).The three-drug regimen (expanded regimen) based on two nucleoside reverse-transcriptase inhibitors (basic regimens) plus a boosted protease inhibitor is recommended. The decision to initiate the type of regimens—either 2-drugs or 3-drugs—depends on the

type of exposure and clinical status of HIV-positive source person (Annex 10). An emergency supply of these drugs should be available in all hospitals dealing with potentially infected material.

Possible side-effects occur mainly at the beginning of the treatment and, however, most side-effects are mild. Adherence information is essential with psychological support and more than 95% adherence is important in order to maximize the efficacy of the medication in PEP. A complete blood count and liver function tests (particularly transaminases) should be performed at the beginning of treatment (baseline) and four weeks later. The exposed person must be clinically monitored for the eventual appearance of signs indicating an HIV seroconversion. HIV testing should be done as part of the PEP with an informed consent with standard pre-test and post-test counselling according to national and/or local protocols. Therefore, the HIV test should be done at baseline, three months and again at six months. During the follow-up period, especially the first 6–12 weeks, when most infected persons are expected to show signs of infection, the recommendations for preventing transmission of HIV are to be followed by health-care workers. These include refraining from blood, semen, organ donation and abstaining from sexual intercourse.

In case the person indulges in sexual intercourse during this period, the importance of correct and consistent condom use should be communicated to the individual. Additionally, women should not breastfeed their infants during the follow-up period to minimize risk of transmission to the child.

If the exposed person is unvaccinated or unclear about hepatitis B vaccination status, the full course of hepatitis B vaccine should be given. There is presently no prophylaxis available against hepatitis C.

11

Collection, storage, packaging and transport of biological specimens

Biospecimens may be sent to the testing laboratory for HIV diagnosis (serum or plasma), CD4 T lymphocytes count (whole blood with EDTA), viral load testing (plasma) and HIV drug resistance testing and viral isolation (plasma).

Blood specimens represent the most common analytical sample requirement. Certain techniques and care must be exercised to ensure that an acceptable specimen is collected and to preclude any adverse affect to the patient. In general, the phlebotomy requires the use of a 20-to 22-gauge needle to minimize mechanical haemolysis during aspiration and either a syringe or evacuated tube collection system.

The collection of blood is described as follows:

- Gloves should be worn and sterilized/disposable syringes and needles should be used.
- For avoiding soiling, a big piece of absorbent cotton may be placed below the forearm before commencing venepuncture.
- After collecting 5 ml of blood aseptically, it should be carefully transferred from the syringe into a sterile, plastic leak-proof specimen container, preferably screw-capped.
- The containers should be labelled before commencement of venepuncture.
- If the vial has anticoagulants, then a second person wearing gloves should help in shaking the vial for mixing the blood well with the anticoagulants. The cap should be tightly screwed after the blood has been transferred to the vial.

- After the blood is collected, the tourniquet is removed and needle is withdrawn. The patient is given a dry sterile cotton swab to press over the site of venepuncture. Elbow may be flexed to keep the cotton swab in place till the blood stops. Any bloodspill should be carefully wiped with 70% ethanol.
- All the swabs and cotton pieces are placed in plastic bags for disposal. If the outside of the vial is visibly contaminated with blood, it should be cleaned with 10% freshly prepared sodium hypochlorite solution.
- The blood is allowed to clot for 30 minutes (not more than 2 hours) at room temperature. The clot may be gently broken if necessary using sterile Pasteur pipettes.

Separation of serum

- The collection tube is centrifuged at 1200 g (3000 rpm) for 10 to 15 minutes to separate serum to avoid haemolysis. If no centrifuge is available, the blood with clot may be left in the refrigerator at +4 °C over the night. The clot will retract and get separated from serum.
- Allow the blood to clot for at least 30 minutes before centrifugation.
- The specimen vial is unstoppered, the serum is drawn off by sterile Pasteur pipette and transferred to a sterile plastic screw-capped leak-proof tube.

Storage of serum specimens

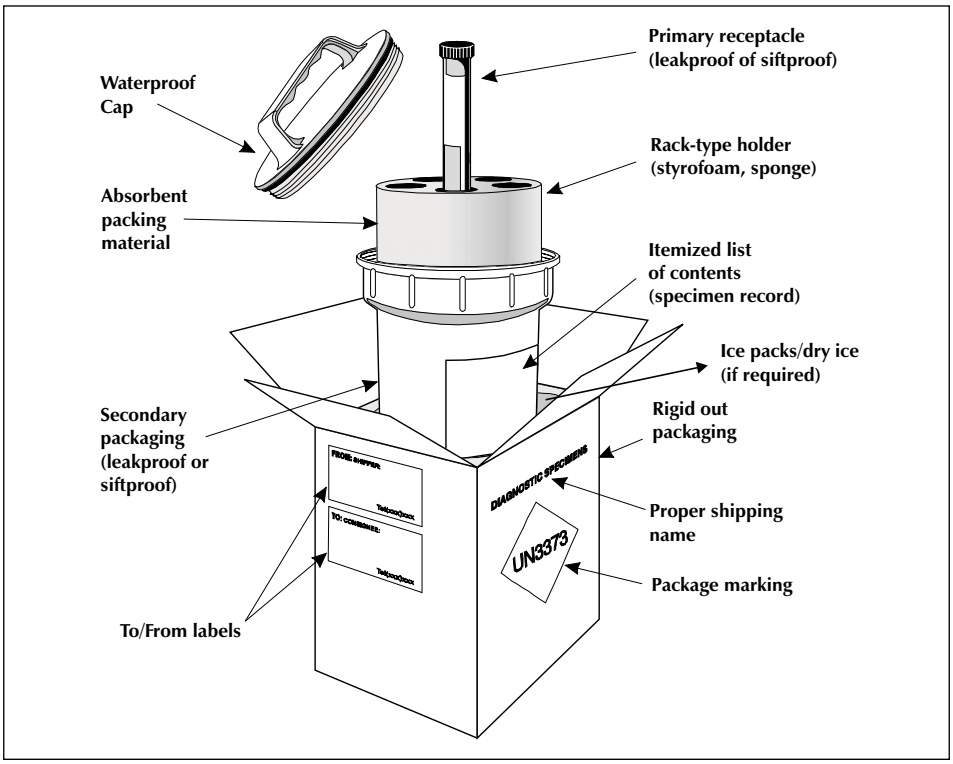
- The sera samples are placed in leak-proof plastic containers in the refrigerator at +4 °C for temporary storage (up to five days).
- The outside of the container is checked for visible contamination with blood which should be cleaned.
- For storage for a long time, deep-freezing at –70 °C is advised and cryovial should be used for deep-freezing purposes.

For plasma or whole blood specimen, K3 EDTA (1.5 ± 0.15 mg/ml blood) tube could be used and after the collection, the tube should be gently inverted at least five times to mix blood and additive. The separation of plasma can be done as in the case of serum. For viral load and HIV drug resistance testing the whole blood specimen should be processed within six hours.

Transport of serum/plasma specimens

- The specimen tube, in which serum/plasma is to be transported, should not have a crack or leakage. Preferably, it should be made of plastic and should be screw-capped with "O"-ring. The outside of the container should be checked for any visible contamination with blood which should be disinfected.
- The tube should be labelled and then placed in a second tightly-capped unbreakable container (Figure 11.1) surrounded by adequate packing material (such as tissue paper, absorbent cotton, etc.) to absorb liquid if leakage occurs accidentally.
- The secondary container should also have a label. This is placed in a thermocol box having ice packs or dry ice to maintain proper cold chain system during transit.
- A proforma with details, i.e. name, age, sex, risk factors, history of previous testing, etc. should accompany the specimen.

Figure 11.1: **Packaging infectious substances for the shipment**



The shipment of the infectious substance is regulated by the Transportation of Dangerous Goods regulations and the International Air Transport Association (IATA) dangerous goods regulations (DGR). The following guidelines provide information for classifying infectious substances for transportation and ensuring their safe packaging.

Infectious substances

For the purposes of transport, infectious substances are defined as substances which are known or are reasonably expected to contain pathogens. Pathogens are defined as microorganisms and other agents such as prions, which can cause disease in humans or animals. Infectious substances are divided into two main categories:

1) Infectious substance, Category A

An infectious substance (highly pathogenic) which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans.

Infectious substances meeting these criteria which cause disease in humans or in both humans and animals shall be assigned United Nations number UN 2814 (the proper shipping name is 'Infectious substance, affecting humans'). The indicative examples of substances that meet these criteria are given in the *Guidance of Regulations of the Transport of Infectious Substances 2007-2008 (WHO)*.

2) Infectious substance, Category B

An infectious substance which contains or is suspected to contain infectious substance that do not meet Category A criteria.

Infectious substances in Category B shall be assigned UN 3373 (the proper shipping name is 'Biological substance, Category B').

Exempt specimens

Human (patient specimens) for which there is minimal likelihood that pathogens are present are not subject to these regulations if the specimen is transported in a packaging which will prevent any leakage and which is marked with the words "Exempt human specimen".

Basic triple packaging system

This system of packaging shall be used for all infectious substances. It consists of three layers as follows:

(1) Primary receptacle: A primary watertight, leak-proof receptacle containing the specimen. The receptacle is packaged with enough absorbent material to absorb all fluids in case of breakage.

(2) Secondary packaging: A second durable, watertight, leak-proof packaging to enclose and protect the primary receptacle(s). Several cushioned primary receptacles may be placed in one secondary packaging, but sufficient additional absorbent material shall be used to absorb all fluid in case of breakage.

(3) Outer packaging: Secondary packagings are placed in outer shipping packagings with suitable cushioning material. Outer packagings protect their contents from outside influences, such as physical damage, while in transit. The smallest overall external dimension shall be 10x10 cm.

Each completed package is normally required to be correctly marked, labelled and accompanied with appropriate shipping documents (as applicable).

For the shipment of the diagnostic specimens collected from the HIV/AIDS patients, the following shipping criteria is applicable: Infectious substance, Category B as per the IATA 2007 requirements. However, these guidelines may be used in conjunction with the policy and regulations of the country concerned.

Table 11.1: IATA shipping requirements for infectious substances in Category B

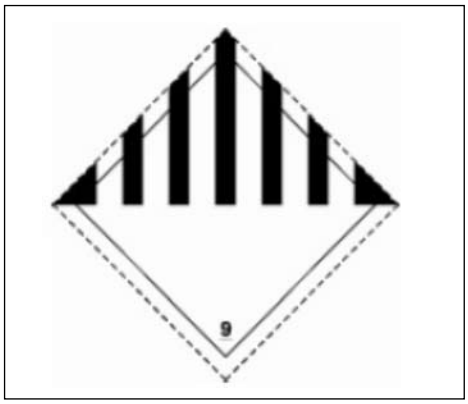
Packaging	• Triple packaging. • The primary and secondary receptacles shall be leak-proof. • The primary receptacle or the secondary packaging shall be capable of withstanding, without leakage, an internal pressure of 95 kPa (0.95 bar). • Absorbent material shall be placed between the primary receptacle(s) and the secondary packaging. • Packing instructions P650. • The volume shipped per package should not exceed 4 kg or 4 l (excluding the ice or dry ice).
Marking	Each package should display the following marking: • Shipper's name, address and telephone number. • The telephone number of a responsible person, knowledgeable about the shipment. • Receiver's name, address and telephone number. • Package labelled with "BIOLOGICAL SUBSTANCE, CATEGORY B" in letters at least 6 mm tall • Label UN3373 in diamond-shaped mark (Figure 11.2) • Temperature storage requirements (optional). • When dry ice is used the label shown in Figure 11.3 should be used.
Documentation	• Packing list. • Proforma invoice. • An import or export permit. • Does not require Shipper's Declaration for Dangerous Goods

Responsibilities of the shipper (consigner)

- Make advance arrangements with the receiver including investigating the need for import/export permits.
- Make advance arrangements with the carrier to ensure:
  - that the shipment will be accepted for appropriate transport.

Figure 11.2: **Label for Biological Substance, Category B**

Figure 11.3: **Hazard label for dry ice**



- that the shipment (direct transport if possible) is undertaken by the most direct routing.
- Prepares necessary documentation, including permits, dispatch and shipping documents.
- Notifies the receiver of transportation arrangements once these have been made, well in advance of the expected arrival time.

Responsibilities of the receiver (consignee)

- Obtain the necessary authorization(s) from national authorities for the importation of the material.
- Provide the sender with the required import permit(s), letter(s) of authorization, or other document(s) required by the national authorities.
- Arrange for the most timely and efficient collection on arrival.
- Should acknowledge receipt to the sender.

Shipments should not be dispatched until

- Advance arrangements have been made between the sender, carrier and receiver.

- The shipper has confirmed with the national authorities that the material may be legally exported.
- The receiver has confirmed with the national authorities that the material may be legally imported.
- The receiver has confirmed that there will be no delay incurred in the delivery of the package to its destination.

Transport of whole blood for CD4/CD8 T lymphocytes count

- The tube containing the specimen should be placed in a leak-proof container (e.g. a sealed plastic bag). This container should be packed inside a cardboard canister containing sufficient material to absorb all the contents should the tube break or leak. Cap the canister tightly. Fasten the request slip securely to the outside of this canister with a rubber band. For mailing, this canister should be placed inside another canister containing the mailing label.
- The specimen should be transported as early as possible after the collection and the test should be performed within the time-frame allowed by instrument manufacturer (e.g. for FACSCount, within 48 hours).
- During the transport of specimens, ambient temperature (20-25 °C) should be maintained and specimens should not be exposed to extreme temperatures that could allow them to freeze or become too hot.
- Temperatures >37 °C might cause cellular destruction and affect flow cytometry measurements. 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 retain the specimen at ambient temperature.

Collection and transport of dried blood spots (DBS)

The DBS specimens are easy to collect in a resource-limited setting and can be stored and shipped to testing facilities without refrigeration. Filter paper used in the collection of DBS for HIV testing is Schleicher and Schuell #903.

The DBS can be collected from a heel-stick (1-4 months, less than 6 kg) or toe-stick (5-10 months, less than 10 kg) or finger stick (larger than 10 kg) or venous blood onto filter paper. The following is the recommended procedure for the collection of the DBS specimen:

- Warm the baby's foot or hand to facilitate blood flow. This can be done by wrapping a hand around the foot while the baby sits in its mother's lap.
- Position the baby with its feet down.
- Clean the baby's foot with disinfectant and let it dry.
- While wearing gloves free of powder, the phlebotomist pricks the baby's foot with a lancet to draw blood.
- The first drop of blood should be wiped away with gauze or cotton wool.
- The phlebotomist should then allow a large drop of blood to collect on the foot before touching it to the circle on the filter paper. The circle should be filled completely by the blood drop and at least two circles should be filled per card.
- The foot should then be cleaned with disinfectant.
- Specimen should be stored horizontally out of direct sunlight for at least three hours. Once dried, the specimens are stored in sealable plastic bags with desiccant packets and a humidity card and then transport it to the testing laboratory. If not sent that day, samples should be refrigerated, though they need not be refrigerated during transport.

Suggested further reading

(1) Balakrishnan P, Solomon S, Mohanakrishnan J, Cecelia AJ, et al. A reliable and inexpensive EasyCD4 assay for monitoring HIV-infected individuals in resource-limited settings. J Acquir Immune Defic Syndr. 2006; 43:23-6.

(2) Pattanapanyasat K, Sukapirom K, Kowawisatsut L, Thepthai C.New BD FACSCount CD4 reagent system for simultaneous enumeration of percent and absolute CD4 T-lymphocytes in HIV-1-infected pediatric patients. Cytometry B Clin Cytom. 2008;74 Suppl 1:S98-106.

(3) Pattanapanyasat K, Phuang-Ngern Y, Sukapirom K, Lerdwana S, et al. Comparison of 5 flow cytometric immunophenotyping systems for absolute CD4+ T-lymphocyte counts in HIV-1-infected patients living in resource-limited settings. J Acquir Immune Defic Syndr. 2008 Dec 1;49:339-47.

(4) Kannangai R, Ramalingam S, Prakash KJ, Abraham OC, et al. Molecular confirmation of human immunodeficiency virus (HIV) type 2 in HIV-seropositive subjects in south India. Clin Diagn Lab Immunol. 2000 ;7:987-9.

(5) Syed Iqbal H, Balakrishnan P, Murugavel KG, Suniti S. Performance characteristics of a new rapid immunochromatographic test for the detection of antibodies to human immunodeficiency virus (HIV) types 1 and 2.J Clin Lab Anal. 2008; 22:178-85.

(6) WHO Guidance on regulations for the Transport of Infectious Substances 2007–2008.2007. Available at http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2cc.pdf (accessed on 12April 2009).

(7) Post-exposure prophylaxis to prevent HIV infection. Joint WHO/ILO guidelines on post-exposure prophylaxis (PEP) to prevent HIV infection. 2007. Available at http://whqlibdoc.who.int/publications/2007/9789241596374_eng.pdf (accessed on 12April 2009).

(8) WHO Guidelines on Improving the diagnosis and treatment of smear-negative pulmonary and extrapulmonary tuberculosis among adults and adolescents Recommendations for HIV-prevalent and resource-constrained settings. 2007. Available at http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/smear_neg_and_extrapulmTb.pdf (accessed on 12April 2009).

(9) Guidelines for Good Clinical Laboratory Practices (GCLP) Indian Council of Medical Research, New Delhi. 2008. Available at http://icmr.nic.in/guidelines/GCLP.pdf (accessed on 12 April 2009).

(10) WHO-SEARO Guidelines on Establishment of Accreditation of Health Laboratories. 2007. Available at http://www.searo.who.int/LinkFiles/Publications_SEA-HLM-394.pdf (accessed on 12 April 2009).

(11) Akinola NO, Olasode O, Adediran IA, Onayemi O, et al. The search for a predictor of CD4 cell count continues: total lymphocyte count is not a substitute for CD4 cell count in the management of HIV-infected individuals in a resource-limited setting. Clin Infect Dis. 2004 15;39:579-81.

(12) Stevens W, Sherman1 G, Downing R, Parsons LM, et al. Role of the Laboratory in Ensuring Global Access to ARV Treatment for HIV-Infected Children: Consensus Statement on the Performance of Laboratory Assays for Early Infant Diagnosis. Open AIDS J. 2008;2:17-25.

(13) Marc Mendelson, Diagnosing tuberculosis in HIV-infected patients: challenges and future prospects. British Medical Bulletin 2007 81-82(1):149-165.

(14) Guidelines: CDC Guidelines for performing single-platform absolute CD4+ T-cell determinations with CD45 gating for persons infected with human immunodeficiency virus. MMWR, 2003; 52(No. RR 02).

(15) Guidelines: CDC Revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). MMWR 1997; 46 (No. RR-2).

(16) Guidelines : CDC Guidelines for National Human Immunodeficiency Virus Case Surveillance, Including Monitoring for Human Immunodeficiency Virus Infection and Acquired Immunodeficiency Syndrome. MMWR. December 10, 1999 / 48 (RR 13); 1-28.

(17) Guidelines: Guidelines for preventing HIV, HBV and other Infections in the Health care setting. WHO-SEARO Publication, New Delhi, India 1999.

(18) Guidelines: Guidelines on Prevention and control of Hospital associated Infections. WHO, New Delhi, (SEA-HLM-343), 2002.

(19) Guidelines: CDC Updated U.S. Public Health Service Guidelines for the Management of Occupational Exposures to HBV, HCV, and HIV and Recommendations for Postexposure Prophylaxis. MMWR.2001.

(20) Guidelines. Guidelines on Standard Operating Procedures for Microbiology. WHO New Delhi, India. 2001.

(21) Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections. WHO – New Delhi. 2001.

(22) Harrigan PR, Montaner JS, Wegner SA, Verbiest W, *et al*. World-wide variation in HIV1 phenotypic susceptibility in untreated individuals: biologically relevant values for resistance testing. *AIDS* 2001; 15: 1671-7.

(23) Kumari S, Bhatia R. Guidelines for Peripheral and Intermediate Laboratories in Quality Assurance in Bacteriology and Immunology. WHO Regional Publication, South-East Asia Series No. 28, New Delhi, India. 2003.

(24) Kumarasamy N, Suniti Solomon, Timothy P. Flanigan, Hemalatha R, *et al*. Natural History of Human Immunodeficiency Virus Disease in Southern India. *Clinical Infectious Diseases* 2003;36:79-85

(25) Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections. WHO–New Delhi, India. 2001.

(26) Report: Role of Health Laboratories in the Implementation of the "3 by 5" Initiative. SEARO Newsletter on Quality Assurance in Blood Safety and Health Laboratory Services, New Delhi, India. January 2004. Vol. 4, No.1.

(27) WHO consultation on technical and operational recommendations for scale-up of laboratory services and monitoring HIV antiretroviral therapy in resource –limited settings. Geneva, 13-15 December 2004.

(28) Balakrishnan P, Kumarasamy N, Kantor R, Solomon S, Vidya S, Mayer KH, Newstein M, Thyagarajan SP, Katzenstein D, Ramratnam B. HIV type 1 genotypic variation in an antiretroviral treatment-naive population in southern India. AIDS Res Hum Retroviruses. 2005 Apr; 21(4): 301-5.

(29) Balakrishnan P, Solomon S, Kumarasamy N, Mayer KH. Low-cost monitoring of HIV infected individuals on highly active antiretroviral therapy (HAART) in developing countries. Indian J Med Res. 2005; 121: 345-55.

(30) WHO Technical Information: CD4+ T-cell enumeration technologies. 2004. Available at http://www.who.int/diagnostics_laboratory/publications/en/cd4_is_draft.pdf (accessed on 12 April 2009).

Annex 1

Suggested sources of availability of laboratory equipment and supplies

The following websites are suggested only for information and not recommended by WHO. Programme managers are encouraged to get more information from other sources also.

www.axygen.com; www.bio-rad.com;www.bd.com;
www.beckmancoulter.com; www.abbottdiagnostics.com;
www.biomerieux.com; www.tranasia.co.in; www.sysmex.com;
www.ambalayellowpages.com/5.htm; www.kendro.com;
www.dmoz.org/Science/Instruments_and_Supplies/Laboratory_Equipment;
www.wheecon.com; www.roche.com; www.guavatec hnologies.com;
www.span.co.in; www.orthoclinical.com; www.labindia.com;
www.catalogs.indiamart.com/category/scientific-instruments.html;
www.ifcc.org/ejifcc/vol15no1/150102200401n.htm;
www.trade-india.com/dyn/gdh/catalog/hp/1919/catalog.htm;
www.trade-india.com/dyn/gdh/eyp/Industrial_Supplies/Laboratory_
Glassware_and_Equipment/;
www.noor-scientific.com/bio/thermo.htm ;
www.indiayellowpagesonline.com/a-z/l/2.htm;
www.biowire.com; www.labsafety.com;www.labfurnishers.com;
www.remelinc.com/products/clinical/ laboratory_supplies.cfm;
www.who.amds.com

Sources of EQAS

www.cms.hhs.gov/clia/ptlist.pdf; www.phppo.cdc.gov/mlp/EQA_qlist.asp;
www.who.int/eht/Main_areas_of_work/DIL/Lab_Tech/External_Quality_
Assessment.htm; www.aab.org; www.nrl.gov.au/;
www.cap.org; http://www.digitalpt.com

Bio-specimen shipping training (IATA)

www.worldcourier.com; www.saftpak.com

Online courses (free)

http://ccghe.org/western_blot/player.htm

http://ccghe.org/cd4/player.htm

http://www.bdbiosciences.com/immunocytometry_systems/support/training/online/ITF/start.html

Annex 2

Collection and storage of clinical specimens

Lab. test	Specimen	Recommended activities	Specimen integrity at RT	Short-term storage	Long-term storage	Factors associated with erroneous results
HIV testing and other serology	Serum	–	–	Serum should be separated within 4 hours if stored at RT and 24 hours if stored at 2-8 ^{0}C	–20 ^{0}C or colder	Hemolysis, hyperlipemic, contamination
CD4	Whole blood	*	*K2 EDTA or heparin	Preferably 25±2 ^{0}C	–	Clot haemolysis, storage more than recommended contamination
Viral load	Plasma		K2 EDTA	Preferably 25±2 ^{0}C Plasma should be separated within 6 hours	–70 ^{0}C or colder	Use of unsterile storage vials, fluctuation in freezer temperature. More than 3 freeze-thaw cycle
Haemoglobin complete blood count	Whole blood		K2 EDTA	Preferably 25±2 ^{0}C	–	Clot, Hemolysis
Blood chemistry, sugar, lactate	Plasma	Sodium flouride	Preferably 25±2 ^{0}C Plasma should be separated within 30 min	At 2-8 ^{0}C for same day testing	–20 ^{0}C or colder	Clot, delayed specimen processing. For lactate fasting specimen is recommended
Blood chemistry Serum electrolytes	Serum	–	Preferably 25±2 ^{0}C Serum should be separated within 30 minutes	At 2-8 ^{0}C for same day testing	–20 ^{0}C or colder	Delayed specimen processing
Blood chemistry LFT, RFT, etc.,	Serum	–	Preferably 25±2 ^{0}C	At 2-8 ^{0}C for same day testing	–20 ^{0}C or colder	Haemolysis
Pregnancy testing	Urine or serum	–	Preferably 25±2 ^{0}C	At 2-8 ^{0}C for same day testing	–	Early morning urine is recommended

Lab. test	Specimen	Recommended activities	Specimen integrity at RT	Short-term storage	Long-term storage	Factors associated with erroneous results
OI diagnosis	Sputum CSF, serum, swabs, stool etc.	–	Preferably 25±2 ^{0}C	At 2-8 ^{0}C Stool should be processed and tested immediately for parasites	–	Improperly collected, transported specimen

Annex 3

Procedures carrying potential risks of HIV, HBV and other bloodborne agents

Procedure	Person at risk	Mode of transmission
Collection of blood sample	Patient	Contaminated needle Contaminated hand or gloves of health worker Skin puncture by needle or broken specimen container Contamination of hands by blood
Transport of specimens (within laboratory)	Laboratory personnel Transport worker	Contaminated exterior of specimens container Broken container Spill or splash of specimen
HIV serology and virology	Laboratory personnel	Skin puncture or contamination of skin or Mucous membrane Contaminated exterior of specimen container Contaminated work surface Spill or splash of specimen Broken specimen container Perforated gloves
Cleaning and maintenance	Laboratory personnel Support staff	Skin puncture or skin contamination Spills or splashes Contaminated work surface
Waste disposal	Laboratory personnel Support staff Transport worker	Contact with contaminated waste Puncture wounds and cuts
Shipment of specimens (to other centers)	Public transport worker Postal worker Public	Broken or leaking specimen containers and packages

Annex 4

Summary of CD4 T lymphocytes enumeration technologies: flow cytometry

Parameter	Double platform[a]	Single platform	
		Volumetric[b]	Bead-based[c]
Instruments, manufacturers	• Partec GmbH (Munster, Germany)[d] • Becton Dickinson (California, USA)[e] • Coulter Corporation (Florida, USA)e	• Partec GmbH (Munster, Germany)[d] • Guava Technologies (California, USA)	• Becton Dickinson (California, USA)[e] • Coulter Corporation (Florida, USA)[e]
Cost of instrument (US$)	20 000–95 000	20 000–70 000	20 000–95 000
Cost of reagents/test (US$)	3–11	2–10	8–25
Specimen	Whole blood	Whole blood	Whole blood
Results	• Absolute CD4 and CD8 count • CD4% and CD8% • CD4/ CD8 ratio	• Absolute CD4 and CD8 count • CD4% and CD8% • CD4/ CD8 ratio	• Absolute CD4 and CD8 count • CD4% and CD8% • CD4/ CD8 ratio
Throughput (samples/day)	Up to 200	Up to 50	Up to 200

Parameter	Double platform[a]	Single platform	
		Volumetric[b]	Bead-based[c]
Advantages	• Accurate pipetting less crucial • One tube assay possible without QC problems • EQAS available	• No need for extra beads or haematology analyzer • Protocols for aged Samples available • EQAS available	• No need for haematology analyzer • Protocols for aged samples available • EQAS available
Disadvantages	• Requires the use of a haematology analyser • Fresh samples needed in order to obtain absolute counts	• Requires accurate pipetting technique. • Internal QC for pipetting requires two tubes assay. • Limited validation data available	• Requires accurate pipetting technique • Some equipments are not suitable for paediatric use (e.g. BD FACSCount) • Internal QC for pipetting requires two tubes assay • Beads are expensive and require careful handling

a) Any flow cytometer from any of the three manufacturers can operate with this method to provide absolute counts. The results of flow cytometry are combined with those from haematology in order to calculate absolute counts.

b) Volumetric instruments have the inherent hardware property of measuring the volume of the sample, providing direct absolute counts without the use of haematology analysers or beads.

c) Any flow cytometer from any of the three manufacturers can operate with this method to provide absolute counts.

d) Instruments from this manufacturer, including the cyflow, remain to be validated as volumetric absolute CD4 T –cell counters by independent investigators in multicentre studies.

e) Economic flowcytometry approach (Pan Leukogating –PLG) is possible with these instruments, either with beads (single platform) or without beads (double platform).

Annex 5

Summary of CD4 T lymphocytes enumeration technologies: dedicated and manual assays

Parameter	Dedicated technology			Manual assay
	FACSCount	Cyflow counter	PointCare NOW	Dynabeads
Manufacturer	Becton Dickinson (California, USA)	Partec GmbH (Munster, Germany)	Pointcare Technologies Inc., MA, USA)	Dyna AS (Oslo, Norway)
Instrumentation	Dedicated CD4 counter	Dedicated CD4 counter	Dedicated CD4 and CD4% counter	Magnet Haemocytometer Light or fluorescence microscope
Assay principle	Flow cytometry	Flow cytometry	Flow cytometry	Direct observation of immunocaptured cells
Detection system	Fluorochrome-labelled anti-CD3,CD4 and CD8 Mab	Fluorochrome – labeled anti-CD4, (CD45,CD3, CD8) MAb	Anti-CD4 MAb conjugated with gold nanoparticles.	Magnetic beads conjugated to anti anti-CD4 and CD8 MAb
Specimen	Whole blood	Whole blood	Whole blood	Whole blood
Results	• Absolute CD4 and CD8 counts • CD4/CD8 ratio • CD4%and CD8%	• Absolute CD4 count	• Absolute and % CD4 count • Absolute WBC count • Absolute and % lymphocyte count	• Absolute CD4 count • Absolute CD8 count • CD4/CD8 ratio
Correlation with flow cytometry [a] (r value)	0.93–0.98 (several international studies)	0.94–0.98 (some international studies)	Not Available	0.94 and 0.96 (several international studies)
Cost of instrument (US$)	28 000	20 000	25 000 – 29 000	1000–10 000 [b]
Cost of reagents/test (US$) [c]	6–20	2	12-15	3–5

Parameter	Dedicated technology			Manual assay
	FACSCount	Cyflow counter	PointCare NOW	Dynabeads
Advantages	• Automated • Fewer steps, less human error and low biohazard risk • Absolute CD4 and CD8 counts • Quick results • EQAS available	• Reagents available at low cost • Quick results • EQAS available	• Simple and no manual gating • No manual sample preparation • Pointcare system	• Simple • Rapid • Absolute CD4 and CD8 counts
Disadvantages	• Expensive reagents • CD4% not reported	• CD4% not reported • Limited validation data available	• Each sample processing takes 17 minutes • No EQAS available • No published data available yet	• Low-throughput • Subjectivity in visual counting • CD4% not reported • No EQAS available
Licensed by USA-FDA	Approved	–	Approved	–

a) The analysis of correlation using linear regression is not appropriate for comparison of methods. Instead, analysis of agreement should be performed. Unfortunately most of the published studies has used this analysis to compare these methods with flow cytometry. The r values are therefore reported there.

b) Depending on whether a light microscope or a fluorescence microscope is used.

c) Equipment cost may vary and reagent cost may decrease substantially in the near future.

Annex 6

Summary of main characteristics of viral load technologies based on Nucleic Acid Testing (NAT)

Company	Abbott		Roche		Bayer		BioMerieux		Primagen	
Assay name	HIV RNA quantitative		Amplicor HIV-1 monitor test		Versant HIV-1 RNA 3.0 assay		NucliSens EasyQ HIV-1		Retina Rainbow	
Type of assay	RT-PCR		RT-PCR		bDNA		RT-NASBA		RT-NASBA	
Dynamic range (copies/ml)	40–1 000 000		50–750 000		75–500 000		50–3 000 000		500–50 000 000	
Specimen type	Plasma		Plasma, dried blood spots		Plasma		Plasma, serum, dried blood spots		Plasma, serum, whole blood, dried blood spots	
Specimen Volume	200–1000 ul		100–500 ul		1000–200 ul		10–2000 ul		200 ul	
Area of HIV genome amplified	Pol		Gag		Pol		Gag		LTR	
HIV-1 subtypes amplified	Group M (subtypes A to G) and Group O		All, plus some HIV-2		Group M (subtypes A to G)		All		All	
Time for result	5 hours		6–7 hours		22 hours		2.5–3 hours		1.5 hours	
Cost/testa	US$ 20–70		US$ 28–90		US$ 125		US$ 38–76		US$ 17–23	
Number of samples/run	21(+3 controls)		9–48		12–168		8–48		96	
Equipment requiredb	Vaccum pump , centrifuge (x2), heat block	LCX analyzer thermal cycler	COBAS Ampliprep, Dead-air box, computer/printer	Safety hood, Heat block (x 2), centri–fuge (x2)	Bayer system 340 (bDNA analyzer, data manage-ment and computer system)	Centrifuge, heat block, water bath, vaccum system	Nucli Sens miniMAG System / Nucli Sens east MAG system	Nucli Sens EasyQ analyzer, Strip centrifuge	Retin alyser, heat block	Computer, centrifuge
Equipment cost(US$)	8500 + LCX analyzer 25 000		10 000 + COBAS ampliprep 30 000		10,000 + Bayer system analyser				23 000	
Licensed by USA-FDA	–		Approved		Approved		Approved		–	

Annex 7

Summary of main characteristics of viral load technologies not based on nucleic acid (non-NAT)

Company	Cavidi- ExaVir[a]	Perkin Elmer[a]
Assay Name	**ExaVir Load Quantitative HIV-RT Load kit**	**HIV_{-1} p24 Ultra ELISA amplification system**
Type of Assay	Enzyme immunoassay for quantitation of RT activity	Enzyme immunoassay for quantitation of p24 antigen
Dynamic range (copies/ml)	1 - 3000	400
Specimen type	Plasma	Plasma, serum or cell culture supernatant
Specimen Volume	1000 ul	100 ul
Area of HIV genome amplified	RT activity	P24 antigen
HIV_{-1} subtypes amplified	HIV-1	HIV-1
Time for result	24 hours	6 hours
Cost/test	US$20–50	US$10
Number of samples/ run	30	96
Equipment required[b]	Incubator (33°C), freezer, ELISA reader, computer	Incubator, ELISA reader, refrigerator
Equipment cost (US$)	9000–10 000 (start-up pack includes other necessary equipment and three kits)	7000–9000

[a] These assays need extensive validation with the standard assay.

[b] Both assays require piptettes and vortex mixers.

Annex 8

Good laboratory practices

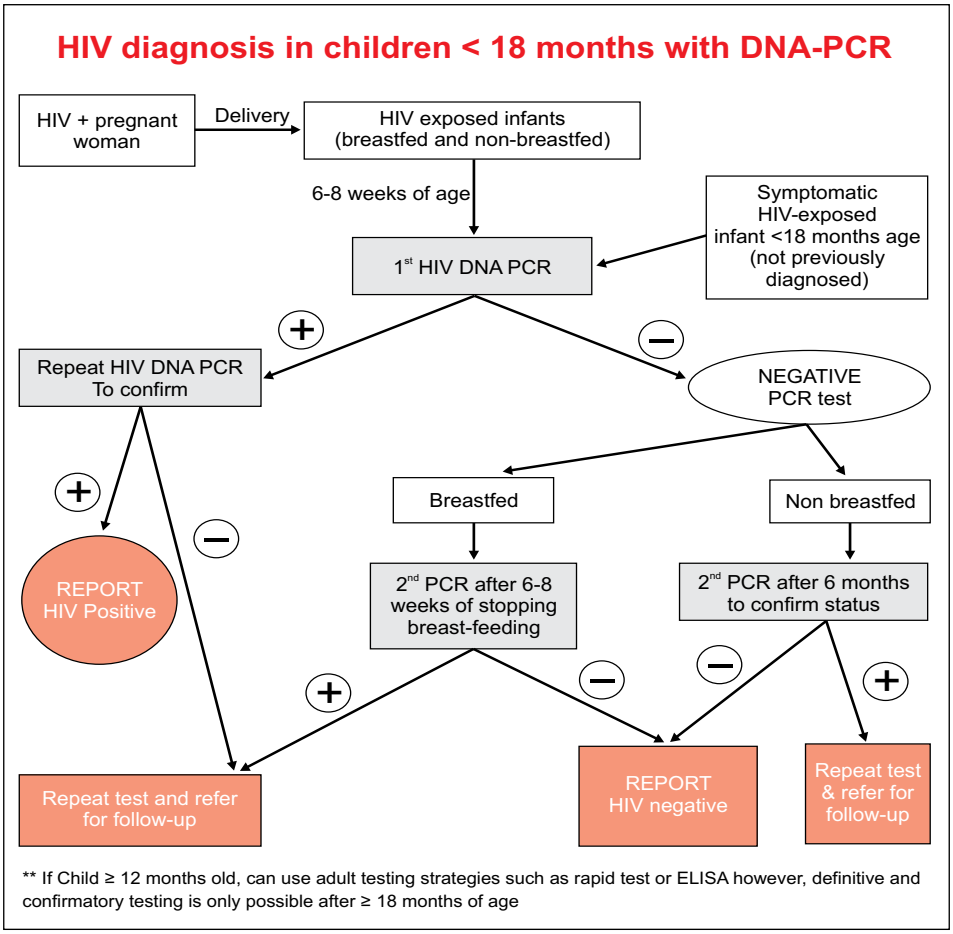
All activities that are performed by the laboratories can be grouped under a common term good laboratory practices (GLP). It indicates performance of all the activities of the laboratory in the best possible way so that the results obtained are of the highest possible accuracy. The GLP encompasses various following factors:

- Laboratory infrastructure
- Human resource
- Equipment and reagents
- Documentation
- General practices:
  - Proper collection of samples
  - Appropriate identification of specimens with special labels on hazardous specimens
  - Prompt transportation to laboratory at appropriate temperature
  - Collection and storage under conditions which prevent deterioration of the sample before the performance of test
  - Accurate performance of test
  - Release of reports after proper scrutiny
  - Delivery of reports to the correct destination in the shortest possible time
  - Cordial relationship with the users

Good laboratory practice (GLP) indicates the performance of all the activities of the laboratory in the best possible way so that the results obtained are of the highest possible accuracy and reliable.

Annex 9

Algorithm for determination of infection status in HIV-exposed children <18 months of age



** If Child ≥ 12 months old, can use adult testing strategies such as rapid test or ELISA however, definitive and confirmatory testing is only possible after ≥ 18 months of age

Annex 10

Contributors and reviewers

Prof. Suniti Solomon, MD.
Director, YRG Centre for AIDS Research and Education(YRG CARE)
Voluntary Health Services Hospital Campus, Taramani
Chennai – 600 113, India
E-mail : suniti@yrgcare.org

Prof. Kovit Pattanapanyasat, Ph.D.
Center of Excellence for Flow Cytometry
Faculty of Medicine
Siriraj Hospital, Mahidol University
Bangkok, Thailand
E-mail: grkpy@mahidol.ac.th

Dr Ramesh Paranjape, Ph.D.
Director
National AIDS Research Institute(NARI)
G-73, M.I.D.C.
Bhosari, Pune-411 026, India
E-mail: rparanjape@nariindia.org

Prof. V. Ravi, MD.
Head
Department of Neuroviroloy
National Institute of Mental Health and Neurosciences (NIMHANS)
Bangalore – 560029, India
E-mail: vravi@nimhans.kar.nic.in

Prof.G. Sridharan,Ph.D.
Head
Department of Clinical Virology
Christian Medical College Hospital
Vellore - 632 004, India
E-mail: g_sridharan@yahoo.com

Dr Madhuri Thakar, Ph.D.
Scientist 'D'
Department of Immunology
National AIDS Research Institute (NARI)
G-73, M.I.D.C.,Bhosari
Pune- 411 026, India
Email: madhurithakar@hotmail.com

Dr Soumya Saminathan, MD.
Deputy Director (Senior Grade)
Division of AIDS Tuberculosis Research Centre,Chetpet, Chennai, India
E-mail : doctorsoumya@yahoo.com

Dr P. Balakrishnan,Ph.D.
Laboratory Manager (Chief/Quality)/
Associate Professor of Microbiology
YRG Centre for AIDS Research & Education
Voluntary Health Services
Hospital Campus
Taramani, Chennai - 600 113, India
E-mail : bala@yrgcare.org

Dr N.Kumarasamy, MBBS, Ph.D.
Chief Medical Officer
YRG Centre for AIDS Research and Education(YRG CARE)
Voluntary Health Services
Hospital Campus
Taramani, Chennai-600 113, India
E-mail: kumarasamy@yrgcare.org

Dr Rajesh Bhatia, MD.
Regional Adviser, Blood safety and Clinical Technology
WHO/South-East Asia Regional Office
IP Estate, Ring Road
New Delhi 110 002, India
00-91-11-23370804 Extn 26504
E-mail: bhatiaraj@whosea.org

Dr Sandhya Kabra,MD.
ADG,
National AIDS Control Organization,
Ministry of Health & Family Welfare,
Govt Of India, New Delhi, India
Email: sandhyakabra@gmail.com

Prof. Rajesh Kannangai, MD.,PhD.
In-charge Retrovirus Laboratory,
Department of Clinical Virology,
Christian Medical College Vellore 632004,
India.
E-mail: rajeshkannangai@hotmail.com

Dr Hanna Luke, Ph.D.
Scientist 'B'
Division of AIDS Tuberculosis Research
Centre
Chetpet, Chennai, India
E-mail: hannatrc@yahoo.com

Dr KG. Murugavel, Ph.D.
Lab Manager (Technical Operation)/
Associate Professor of Microbiology
YRG Centre for AIDS Research & Education
Voluntary Health Services
Hospital Campus, Taramani, Chennai - 600
113, India. Email: murugavel@yrgcare.org

Dr Sunil Solomon, MBBS, MPH, Ph.D.
Medical Officer & Epidemiologist
YRG Centre for AIDS Research and
Education (YRG CARE)
Voluntary Health Services Hospital Campus,
Taramani, Chennai – 600 113, India.
E-mail: sunil@yrgcare.org

Reviewers

Dr Bharat Ramratnam, MD
Laboratory of Retrovirology
Division of Infectious Diseases
Brown Medical School
Providence, RI 02903, USA
E-mail: BRamratnam@Lifespan.org

Dr Sirirat Likanonsakul
Chief, Immunology and Virology Laboratory
Bamrasnaradura Institute
126 Tiwanon Road
Nonthaburi 11000, Thailand
E-mail: siratlik@health.moph.go.th

Laboratory support is critical in all the areas of HIV diagnosis and management. Diagnosis of HIV infection cannot be established by any means other than blood tests by the laboratory. CD4 lymphocyte count is a prerequisite for the initiation of antiretroviral therapy and for monitoring treatment outcome. Both immunological and microbiological monitoring of antiretroviral therapy is therefore exclusively dependent on an efficient laboratory service. While laboratory support to HIV/AIDS programmes is very important, the infrastructure, expertise and networking require strengthening in most countries of our Region. These Guidelines aim to assist Member Countries in scaling up ART and responding to the rapidly evolving HIV/AIDS epidemic.

World Health Organization
Regional Office for South-East Asia
World Health House
Indraprastha Estate,
Mahatma Gandhi Marg,
New Delhi-110002, India
Website: www.searo.who.int

SEA-HLM-382