TaqMan | RealTime

Molecular Diagnostics

HIV-1 Proviral DNA & RNA RealTime

A Complete Guide
2016

HBRL

Behold the Power of Perseverance

HIV-1 Proviral DNA & RNA RealTime

Specification

Technology	:	Taqman Technology: sequence-specific Probe
Specificity	:	100% amplification efficiency with all the 12 genetic subtypes of the HIV-1 M group (A, B, C, D, F, G, H, J, K) and CRFs (AB, AE, AG)- A universal assay.
Sensitivity	:	98% detection of 1-5 copy of Nucleic Acid
Sample	:	PCR Ready Set-up Sample
Sample Vol	:	25 µl
PCR System	:	CFX96™ RealTime Detection System-IVD BIORAD
Software	:	Bio-Rad CFX Manager IVD Edition 1.6
Data Analysis		
Mode	:	PCR Quantification with Standard Curve
Channel		
Detection	:	FAM for HIV-1 LTR/ HEX for CCR5 gene (IC)

HBRL
MOLECULAR DIAGNOSTIC RESEARCH

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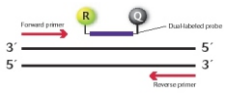
TaqMan | RealTime



Amplify Sequence Specific PCR

CFX96™ Invitro Diagnostic

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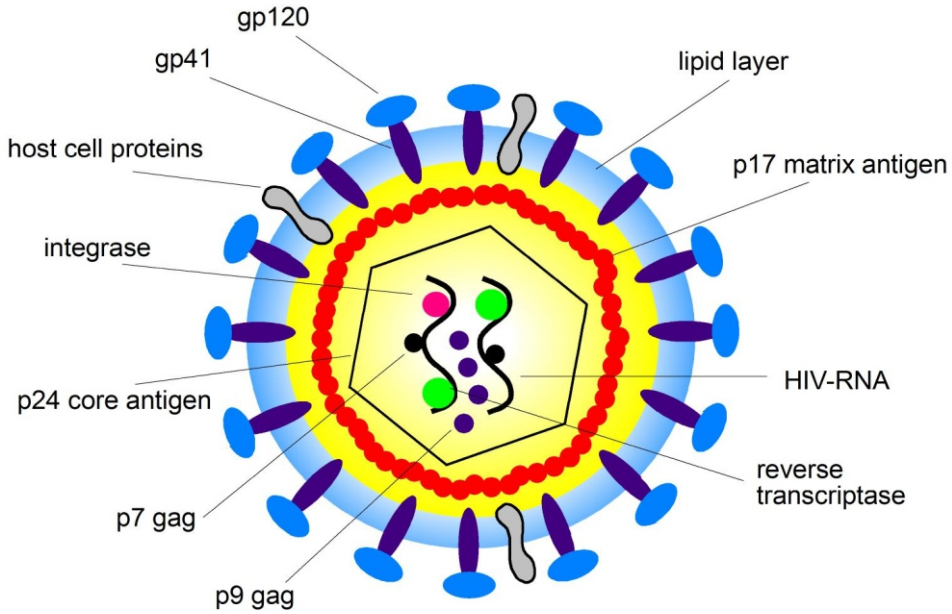
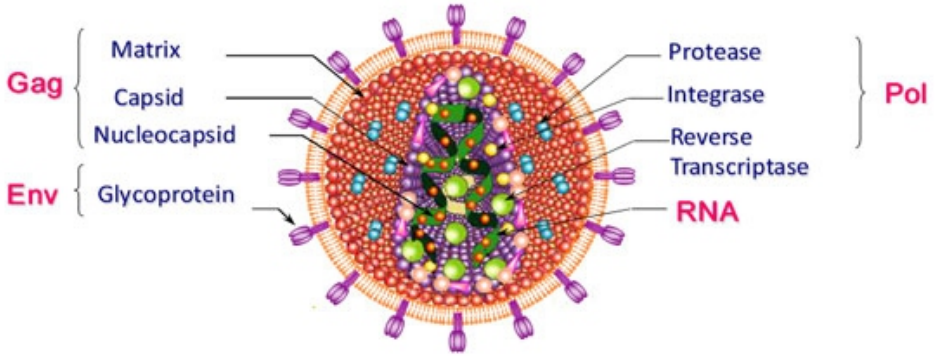
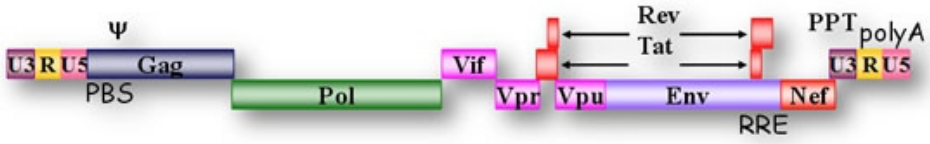


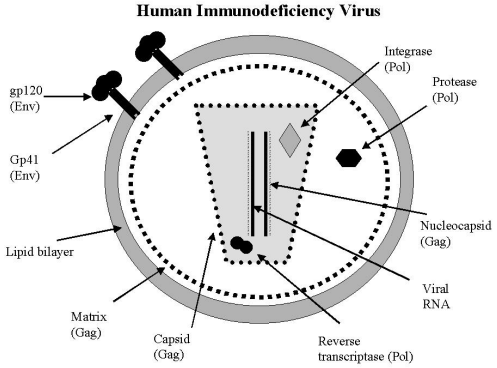




HIV: General Information

Human immunodeficiency virus (HIV) is a lentivirus (a member of the retrovirus family) that causes acquired immunodeficiency syndrome (AIDS), a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive. HIV infects vital cells in the human immune system such as helper T cells (specifically CD4+ T cells), macrophages, and dendritic cells. HIV infection leads to low levels of CD4+ T cells through three main mechanisms: First, direct viral killing of infected cells; second, increased rates of apoptosis in infected cells; and third, killing of infected CD4+ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. When CD4+ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections.

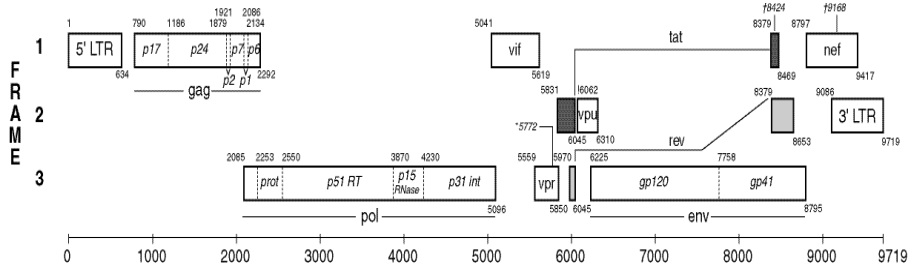
Structure and genome of HIV



SCHEMATIC DIAGRAM OF THE STRUCTURE OF HIV

HIV is different in structure from other retroviruses. It is roughly spherical with a diameter of about 120 nm, around 60 times smaller than a red blood cell, yet large for a virus. It is composed of two copies of positive single-stranded RNA that codes for the virus's nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7, and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by the viral envelope that is composed of two layers of fatty molecules called phospholipids taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle. This protein, known as Env, consists of a cap made of three molecules called glycoprotein gp120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle. Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines against HIV.

GENETIC STRUCTURE OF HIV



HIV-1 GENOME 9749 NUCLEOTIDES

The RNA genome consists of at least seven structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, and INS), and nine genes (gag, pol, and env, tat, rev, nef, vif, vpr, vpu, and sometimes a tenth tev, which is a fusion of tat env and rev), encoding 19 proteins. Three of these genes, gag, pol, and env, contain information needed to make the structural proteins for new virus particles. For example, env codes for a protein called gp160 that is

broken down by a cellular protease to form gp120 and gp41. The six remaining genes, tat, rev, nef, vif, vpr, and vpu (or vpx in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease. HIV

PROTEINS AND THEIR FUNCTIONS

Gene	Protein	Function
Gag MA	p17	Membrane anchoring
Gag CA	p24	Core capsid
Gag NC	p7 and p6	Binds RNA; binds Vpr
Protease	P15	Gag-Pol cleavage and maturation
Reverse transcriptase, RNase H	P66 and p51 heterodimer	Reverse transcription, RNase H activity
Env	Gp120/gp41	External viral glycoproteins, bind to CD4 receptor
Tat	P16/p14	Viral transcriptional activator
Rev	P19	RNA transport
Vif	P23	Promotes virion maturation and infectivity
Vpr	P10–15	Promotes nuclear localization of preintegration complex, inhibits cell division
Vpu (HIV-1)	P16	Promotes extracellular release of viral particles
Vpx (HIV-2)	P12–16	Vpr homologue
Nef	P27/p25	CD4 downregulation
Tev	P28	Tripartite Tat-Env-Rev protein

Tropism

The term viral tropism refers to which cell types HIV infects. HIV can infect a variety of immune cells such as CD4+ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and CD4+ T cells is mediated through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells and also with chemokine co-receptors.

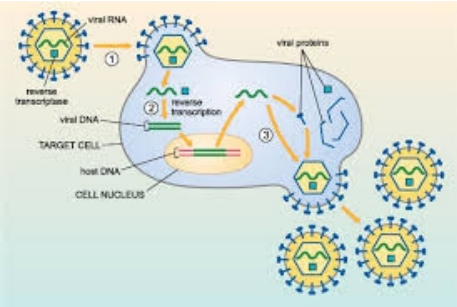
Macrophage (M-tropic) strains of HIV-1, or non-syncitia-inducing strains (NSI) use the β- chemokine receptor CCR5 for entry and are, thus, able to replicate in macrophages and CD4+ T cells. This CCR5 coreceptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. Indeed, macrophages play a key role in several critical aspects of HIV infection. They appear to be the first cells infected by HIV and perhaps the source of HIV production when CD4+ cells become depleted in the patient. Macrophages and microglial cells are the cells infected by HIV in the central nervous system. T-tropic isolates, or syncitia-inducing (SI) strains replicate in primary CD4+ T cells as well as in macrophages and use the α-chemokine receptor, CXCR4, for entry. Dual-tropic HIV-1 strains are thought to be transitional strains of HIV-1 and thus are able to use both CCR5 and CXCR4 as co-receptors for viral entry.

Some people are resistant to certain strains of HIV. For example, people with the CCR5-Δ32 mutation are resistant to infection with R5 virus, as the mutation stops HIV from binding to this co-receptor, reducing its ability to infect target cells.

Sexual intercourse is the major mode of HIV transmission. Both X4 and R5 HIV are present in the seminal fluid, which is passed from a male to his sexual partner. The virions can then infect numerous cellular targets and disseminate into the whole organism. However, a selection process leads to a predominant transmission of the R5 virus through this pathway. How this selective process works is still under investigation, but one model is that spermatozoa may selectively carry R5 HIV as they possess both CCR3 and CCR5 but not CXCR4 on their surface and that genital epithelial cells preferentially sequester X4 virus. In patients infected with subtype B HIV-1, there is often a co-receptor switch in late-stage disease and T-tropic variants appear that can infect a variety of T cells through

CXCR4. These variants then replicate more aggressively with heightened virulence that causes rapid T cell depletion, immune system collapse, and opportunistic infections that mark the advent of AIDS. Thus, during the course of infection, viral adaptation to the use of CXCR4 instead of CCR5 may be a key step in the progression to AIDS. HIV-2 is much less pathogenic than HIV-1 and is restricted in its worldwide distribution. The adoption of "accessory genes" by HIV-2 and its more promiscuous pattern of co-receptor usage (including CD4-independence) may assist the virus in its adaptation to avoid innate restriction factors present in host cells. Adaptation to use normal cellular machinery to enable transmission and productive infection has also aided the establishment of HIV-2 replication in humans.

Replication cycle of HIV



1. Entry to the cell

HIV enters macrophages and CD4+ T cells by the adsorption of glycoproteins on its surface to receptors on the target cell followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell. Entry to the cell begins through interaction of the trimeric envelope complex (gp160 spike) and both CD4 and a chemokine receptor (generally either CCR5 or CXCR4, but others are known to interact) on the cell surface. gp120 binds to integrin α4β7 activating LFA-1 the central integrin involved in the establishment of virological synapses, which facilitate efficient cell-to-cell spreading of HIV.

After HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrase, ribonuclease, and protease, are injected into the cell. During the microtubule-based transport to the nucleus, the viral single-strand RNA genome is transcribed into double-strand DNA, which is then integrated into a host chromosome.

2. Replication and transcription

Shortly after the viral capsid enters the cell, an enzyme called reverse transcriptase liberates the single-stranded (+) RNA genome from the attached viral proteins and copies it into a complementary DNA (cDNA) molecule. The process of reverse transcription is extremely error-prone, and the resulting mutations may cause drug resistance or allow the virus to evade the body's immune system. The reverse transcriptase also has ribonuclease activity that degrades the viral RNA during the synthesis of cDNA, as well as DNA-dependent DNA polymerase activity that creates a sense DNA from the antisense cDNA. Together, the cDNA and its complement form a double-stranded viral DNA that is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by another viral enzyme called integrase.

During viral replication, the integrated DNA provirus is transcribed into mRNA, which is then spliced into smaller pieces. These small pieces are exported from the nucleus into the cytoplasm, where they are translated into the regulatory proteins Tat (which encourages new virus production) and Rev. As the newly produced Rev protein accumulates in the nucleus, it binds to viral mRNAs and allows unspliced RNAs to leave the nucleus, where they are otherwise retained until spliced. At this stage, the structural proteins Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles.

HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 will bind to any appropriate RNA, whereas HIV-2 will preferentially bind to

the mRNA that was used to create the Gag protein itself. This may mean that HIV-1 is better able to mutate (HIV-1 infection progresses to AIDS faster than HIV-2 infection and is responsible for the majority of global infections).

3. Assembly and release:

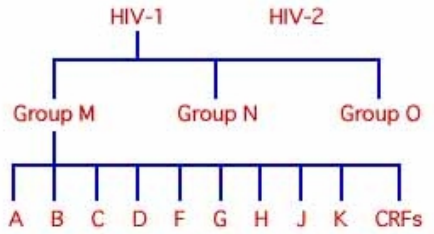
The final step of the viral cycle, assembly of new HIV-1 virons, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation occurs either in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion. This cleavage step can be inhibited by protease inhibitors. The mature virus is then able to infect another cell.

HIV Diversity

HIV-1 & HIV-2

Two types of HIV: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, and from mother to child, and they appear to cause clinically indistinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Worldwide, the predominant virus is HIV-1, and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere.

HIV-1 Subtypes



The strains of HIV-1 can be classified into four groups: the "major" group M, the "outlier" group O and two new groups, N and P. These four groups may represent four separate introductions of simian immunodeficiency virus into humans.

Group O appears to be restricted to west-central Africa and group N - a strain discovered in 1998 in Cameroon - is extremely rare. In 2009 a new strain closely relating to gorilla simian immunodeficiency virus was discovered in a Cameroonian woman, It was designated HIV-1 group P. More than 90 percent of HIV-1 infections belong to HIV-1 group M and, unless specified, the rest of this page will relate to HIV-1 group M only.

Group M have at least nine genetically distinct subtypes (or clades) of HIV-1. These are subtypes A, B, C, D, F, G, H, J and K. Occasionally, two viruses of different subtypes can meet in the cell of an infected person and mix together their genetic material to create a new hybrid virus (a process similar to sexual reproduction, and sometimes called "viral sex"). Many of these new strains do not survive for long, but those that infect more than one person are known as "circulating recombinant forms" or CRFs. For example, the CRF A/B is a mixture of subtypes A and B. The classification of HIV strains into subtypes and CRFs is a complex issue and the definitions are subject to change as new discoveries are made. Some scientists talk about subtypes A1, A2, A3, F1 and F2 instead of A and F, though others regard the former as subsubtypes.

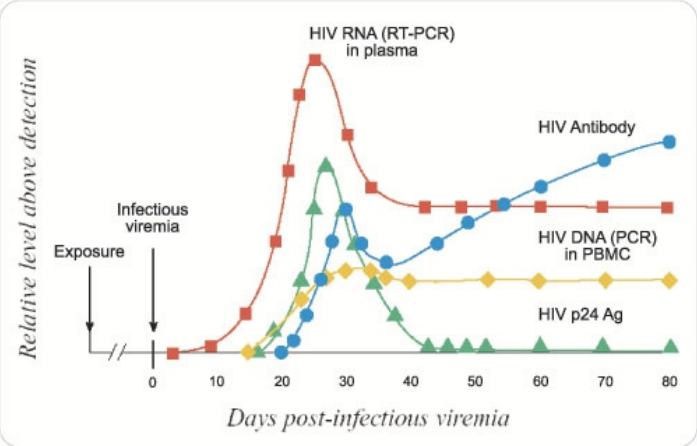
Genetic variability

HIV differs from many viruses in that it has very high genetic variability. This diversity is a result of its fast replication cycle, with the generation of about 1010 virions every day, coupled with a high mutation rate of approximately 3 x 10−5 per nucleotide base per cycle of replication and recombinogenic properties of reverse transcriptase.

Treatment interruption

Although most current HIV-1 antiretroviral drugs were designed for use against subtype B, there is no compelling evidence that they are effective against other subtypes. Nevertheless, some subtypes may be more likely to develop resistance to certain drugs, and the types of mutations associated with resistance may vary. This is an important subject for future research.

Diagnosis



HIV-1 testing is initially by an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to HIV-1. Specimens with a nonreactive result from the initial ELISA are considered HIV-negative unless new exposure to an infected partner or partner of unknown HIV status has occurred. Specimens with a reactive ELISA result are retested in duplicate. If the result of either duplicate test is reactive, the specimen is

reported as repeatedly reactive and undergoes confirmatory testing with a more specific supplemental test e.g., Western blot. Only specimens that are repeatedly reactive by ELISA and positive by IFA or reactive by Western blot are considered HIV-positive and indicative of HIV infection. Specimens that are repeatedly ELISA-reactive occasionally provide an indeterminate Western blot result, which may be either an incomplete antibody response to HIV in an infected person or nonspecific reactions in an uninfected person.

Although IFA can be used to confirm infection in these ambiguous cases, this assay is not widely used. In general, a second specimen should be collected more than a month later and retested for persons with indeterminate Western blot results. Nucleic acid testing (e.g., viral RNA or proviral DNA amplification method) can help diagnosis in every situation. In addition, a few tested specimens might provide inconclusive results because of a low quantity specimen. In these situations, a second specimen is collected and tested for HIV infection. Modern HIV testing is extremely accurate. A single screening test is correct more than 99% of the time. The chance of a false-positive result in standard one-step testing protocol is estimated to be about 1 in 250,000 in a low risk population. Testing post exposure is recommended initially and at six week, three months, and six months. For Confirmation & monitoring of Therapy the Real Time PCR or other Molecular Diagnostic assays are recommended. However Real Time Assays are the first choice due to their simplicity, accuracy & ease of performance.

HIV 1 & 2 Antibody Screen

Antibodies to HIV-1 and HIV-2 are detected by enzyme immunoassay (EIA). Reactive results are confirmed by HIV-1 Western blot. The HIV Western blot identifies antibodies against eight HIV-1 encoded proteins: p18, p24, p31, gp41, p51, p55, p65/66, gp120/p160. Criteria accepted by CDC/ASTPHLD are used for determining a positive HIV Western blot.

These criteria require antibodies against any two of the following HIV-1 proteins: p24, gp41, gp120/160. Specimens showing reactivity to HIV-1 protein(s), but not fulfilling the criteria for a positive result, are reported as Indeterminate. All indeterminate Western blots are further tested in supplemental HIV-1 and HIV-2 specific assays. Specimens showing reactivity to non HIV-1 proteins are not assigned an indeterminate status but are instead reported as "Antibody to non-HIV-1 encoded proteins". A negative Western blot has no detectable bands, i.e. no antibodies reacting to either HIV-1 or non-HIV-1 proteins.

HIV-1 RNA Quantitation

Quantitation of HIV RNA copy number is available to monitor antiviral therapy and to predict disease progression in HIV infected persons. HIV RNA quantitation may be useful to indicate when an HIV infected person should start anti-retroviral therapy and when such therapy should be adjusted. (Alone, this assay is not recommended nor approved for diagnosing HIV infection. However, in conjunction with a positive DNA PCR or a reactive EIA, the RNA quantitation may be diagnostic.) High levels of RNA are found during acute infection and in patients who are more likely to have disease progression. Inhibition of cell-free HIV, as reflected by RNA copy number, is associated with better CD4 response and clinical response in some patient populations.

To quantify HIV RNA, the Clinical Laboratory uses a real-time reverse transcription (RT)-polymerase chain reaction (PCR) amplification platform with enhanced sensitivity and broader dynamic range compared to available commercial assays. The Real-time RT-PCR assay for HIV RNA quantification has been validated against the commercial bDNA and ultrasensitive (US) RT-PCR assays.The dynamic range for HIV RNA detection by Real-Time RT-PCR is 30 to 1,000,000 copies/mL of plasma.

HIV-1 Proviral DNA Detection

The detection of cell associated Human Immunodeficiency Proviral DNA by polymerase chain reaction (PCR) amplification is one of the most sensitive non-serologic methods for confirming HIV infection. In addition to HIV culture, this assay is recommended for confirming HIV infection in the neonate. HIV DNA PCR may also be used as a supplemental test to determine the significance of an indeterminate HIV Western Blot serology result.

HIV-1 Culture

Culture is an extremely sensitive virologic method for documenting HIV infection, especially in neonates whose serologies are complicated by the presence of maternal antibody.

HIV-1 Genotypic Resistance Assay

The assay involves sequencing of the HIV pol gene, after which mutations in the gene can be compared to sequences known to confer resistance to different classes of antiretroviral drugs. The assay is most useful in patients who lose viral suppression on antiretroviral therapy and should be performed before switches in therapy are entertained. Specimen requirement is a 10 mL EDTA tube at room temperature or frozen plasma at -70°C, shipped on dry ice, is also acceptable.

ROUTINE BLOOD COLLECTION PROCEDURE

1. Proper equipment selection and use
2. Proper labeling procedures and completion of laboratory requisitions
3. Identify the additive, additive function, volume, and specimen considerations to be followed for each of the various color coded tubes.

Order Form/ Requisition

A requisition form must accompany each sample submitted to the laboratory. This requisition form must contain the proper information in

order to process the specimen. The essential elements of the requisition form are:

1. Patient's surname, first name, and middle initial.
2. Patient's ID number.
3. Patient's date of birth and sex.
4. Requesting physician's complete name.
5. Source of specimen. This information must be given when requesting microbiology, cytology, fluid analysis, or other testing where analysis and reporting is site specific.
6. Date and time of collection.
7. Initials of phlebotomist.
8. Indicating the test(s) requested.

LAB REQUISITION FORM

Patient Name: _____

Patient ID: _____

Patient Birthdate: _____ Sex: _____

Source of Specimen: _____

Date Collected: _____ Time: _____ Phleb: _____

Physician: _____ Location: _____

Diagnosis: _____

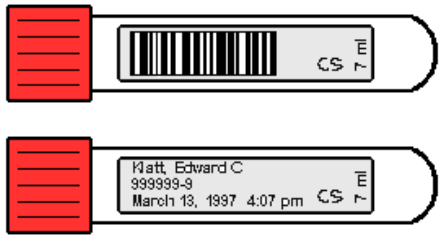
Tests Requested:

LABELING THE SAMPLE

A properly labeled sample is essential so that the results of the test match the patient. The key elements in labeling are:

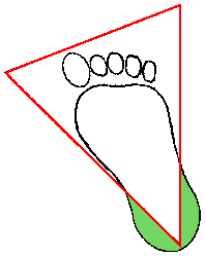
1. Patient's surname, first and middle.
2. Patient's ID number.
3. NOTE: Both of the above MUST match the same on the requisition form.
4. Date, time and initials of the phlebotomist must be on the label of EACH tube.
5. Automated systems may include labels with bar codes.

Examples of labeled collection tubes are shown below:



BLOOD COLLECTION ON BABIES:

1. The recommended location for blood collection on a newborn baby or infant is the heel. The diagram below indicates in green the proper area to use for heel punctures for blood collection.
2. Prewarming the infant's heel (42 C for 3 to 5 minutes) is important to obtain capillary blood gas samples and warming also greatly increases the flow of blood for collection of other specimens. However, do not use too high a temperature warmer, because baby's skin is thin and susceptible to thermal injury.



SAMPLE COLLECTION ON DRIED BLOOD SPOT

Preparation of DBS: Dried blood spot strips were prepared by spotting 100 µl of whole blood onto a Whatman FTA card (4 spots per card). Filter papers were air dried overnight at room temperature and stored at 4°C in plastic sealed bag with a silica desiccant until they were processed. The DBS strips were transported at regular intervals (every 15 to 20 days) in plastic sealed bags at room temperature, followed by storage at 4°C until assay performance.

Whatman FTA Technology

- Whatman FTA cards simplify the isolation, purification and storage of nucleic acids for a variety of research and diagnostic applications. Capture nucleic acid in one easy step
- Captured nucleic acid is ready for downstream applications in less than 30 minutes
- DNA collected on FTA Cards is preserved for years at room temperature
- FTA Cards are stored at room temperature before and after sample application, reducing the need for laboratory freezers
- Suitable for virtually any cell type

Recommended FTA Card: FTA Classic Card (Cat WB 120205)

WESTERN BLOT ASSAY

Sample collection for Western blot assay

For in-vitro detection of antibodies to HIV-1/HIV-2 in human serum or plasma following tubes can be used

For Plasma: Lavender (purple) top (EDTA whole blood) or DBS

Method:

Purple top (EDTA whole blood)

↓ Gently invert to mix

↓

Centrifuge ASAP, transfer supernatant, and discard red cells

↓

Label new tube with owner, Patient ID, and as EDTA PLASMA

↓

Submit this sample as PLASMA

HIV-1 PROVIRAL DNA QUALITATIVE REALTIME

Sample collection for HIV-1 Proviral DNA RealTime

For Whole Blood: Lavender/Purple top (EDTA whole blood) or DBS Method:

Lavender/Purple top (EDTA whole blood)

↓

Gently invert to mix

↓

Label tube with patient ID, and as EDTA WHOLE BLOOD (Do not centrifuge)

↓

Submit this sample for PCR, qPCR tests, as EDTA WHOLE BLOOD.

HIV-1 RNA QUANTITATIVE REALTIME

Sample collection Quantitative measurement of HIV-1 RNA in plasma.

For Plasma: Lavender (purple) top (EDTA whole blood) or DBS Method:

Draw two Purple top (EDTA whole blood)

↓

Gently invert to mix

↓

Centrifuge 800 x g for 15 min within 6 hours, transfer 3.5ml Plasma to screw cap transport Tube

↓

Label new tube with owner, Patient ID, and as PLASMA, store at -20°C

↓

Submit this sample as PLASMA

Purple top (EDTA whole blood)

Principle of Taqman Real-Time PCR

The robust assay exploits the so-called Taqman principle. During PCR, forward and reverse primers hybridize to a specific sequence product. A TaqMan probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR product. A Taq polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. The reporter dye and quencher dye are separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

HIV-1 Proviral DNA Qualitative Realtime

Introduction

Quantification of human immunodeficiency virus type-1 (HIV-1) proviral DNA is increasingly used to measure the HIV-1 cellular reservoirs, a helpful marker to evaluate the efficacy of antiretroviral therapeutic regimens in HIV-1–infected individuals. Furthermore, the proviral DNA load represents a specific marker for the early diagnosis of perinatal HIV-1 infection and might be predictive of HIV-1 disease progression independently of plasma HIV-1 RNA levels and CD4+ T-cell counts. The high degree of genetic variability of HIV-1 poses a serious challenge for the design of a universal quantitative assay capable of detecting all the genetic subtypes within the main (M) HIV- 1 group with similar efficiency. Here, we developed a highly sensitive real-time PCR protocol that allows for the correct quantification of virtually all group-M HIV-1 strains with a higher degree of accuracy compared with other methods. The protocol involves three stages, namely DNA extraction/lysis, cellular DNA quantification and HIV-1 proviral load assessment. Owing to the robustness of the PCR design, this assay can be performed on crude cellular extracts, and therefore it may be suitable for the routine analysis of

clinical samples even in developing countries. An accurate quantification of the HIV-1 proviral load can be achieved within 1 day from blood withdrawal.

In HIV-1 infection, the determination of the number of cell associated HIV-1 DNA copies (HIV-1 proviral load) by real-time PCR is employed not only for research purposes but also for evaluating the effects of antiretroviral therapy on the viral reservoirs. Furthermore, a high HIV-1 proviral DNA load following primary infection has been suggested to be predictive of rapid disease progression independently of plasma viremia and CD4+T-cell counts.

Real-time PCR offers several important advantages over conventional quantitative PCR methods by substantially reducing labor and costs because a single PCR run is sufficient to accurately quantify target DNA without any post-amplification steps. Moreover, the absence of post-amplification manipulation steps greatly reduces the risk of inter-sample contamination and eliminates the need for employing radioactive labels or other hazardous reagents. Finally, by virtue of its high-throughput format, this system is well suited for automatization and use in the routine clinical diagnostic setting. Here, we developed a real-time PCR protocol for the quantification of the HIV-1 DNA load that can be applied equally well to experimental and clinical settings. Although a very limited number of variant isolates may still escape accurate quantification, this protocol is able to correctly measure HIV-1 DNA from a wide panel of viral isolates of diverse genetic subtypes.

Infant Diagnosis

HIV-infection diagnostics in children born from HIV-infected mothers is difficult due to the fact that mother antibodies to HIV persist in such children blood for a long time. But not every child born from the infected mother is infected with HIV, in spite of the fact that children are subjected

to high risk of HIV infection in the intrauterine period, during the delivery and breast feeding. If no preventive measures are taken, the risk of mother-to-child HIV transmission in children makes 20-45 percent. Today effective measures intended at prevention of vertical HIV transmission can reduce risk to 1-2 percent.

The problem of earlier HIV-infection diagnostics in newborns was solved several years ago with development of molecular-genetic methods that allow detection of HIV genome fragments in the peripheral blood at early infection stages. The evidence was obtained that the HIV provirus DNA is determined by the age of one month in the majority of children and practically in all - by the age of 6 months. Based on these data it is recommended to conduct the polymerase chain reaction (PCR) for HIV provirus DNA for the first time within 48 hours after birth and on the 6-8th week of the child life irrespective of the result of the first examination. The final decision about HIV-infection presence in child is made not later than the age of 6 months of the child.

Assay design.

The primer and Taqman probe were designed for a selected a region of the HIV-1 LTR which is highly conserved among all circulating group-M HIV-1 subtypes. The selected region is completely conserved in about 96% of the HIV-1 sequences present in the NCBI database. We have developed an HIV-1 group M-specific quantitative realtime PCR assay that measures the HIV-1proviral DNA load with a similar degree of sensitivity and accuracy regardless of the viral genetic subtype. The success of this protocol for the quantification of HIV-1 proviral DNA relies on the optimized design of primers and probe, which ensures the correct quantification of virtually all circulating group-M HIV-1 strains. Moreover, this assay is so robust that it can quantify DNA derived from a crude lysate with the same degree of accuracy as with purified DNA.

DNA extraction

In standard PCR assays, a critical requirement is that the DNA must be free of contaminants such as hemoglobin, polysaccharides, alcohol or high salt concentrations, as these may interfere with the PCR. In our protocol, we employ Sigma Aldrich, GeneElute, (Cat. no. NA2020), Particular attention was paid on DNAse and RNAse free materials.

HIV-1 DNA real time PCR assay

A series of dilutions of HIV-1 DNA standard was included in each experiment in order to generate an external standard curve. The PCR mixture (total volume 20 µl) in nuclease free water contained MyTaq™ HS DNA Polymerase, final concentrations of 3mM MgCl2 and 0.5µM of each primer and 5µl of purified DNA or negative control. All samples were analysed in duplicate. The amplification protocol for HIV-1 on the CFX96™ (BIORAD) was as follows: a 5 min denaturation step at 95°C for polymerase activation, of 45 cycles consisting of 15 seconds at 92°C, 1 minute at 65°C. The fluorescence was measured at the end of each elongation step. A fragment from the CCR5 gene was amplified in parallel with the HIV-1 LTR gene to quantify the total number of CD4+ cells. Detection involves CFX96TM and CFX96 deepWell Real-Time PCR detection system (IVD)-BIORAD using TaqMan probe.

HIV-1 Plasma RNA Qualitative &Quantitative Realtime

HIV-1 RNA Realtime PCR assay:Real time quantitative PCR using Taqman technology. SPECIFICITY: Assay is designed with specific primers and probe for in vitro quantification of most common HIV-1 genotypes against the Ltr gene.LIMIT OF DETECTION: 40 copies/ml.

The Reagents constitute a ready to use system for detection and quantification.The Specific Master mix contains reagents and enzymes for the specific amplification of HIV-1 and for the direct detection of the specific amplicon in fluorescence channel FAM of the CFX96™

RealTime (BIORAD) & the Reference gene on HEX. External positive Standards (HIV S 1-5) are allows the determination of the gene load.

RNA Extraction

Isolate ll Bodyfluid RNA Kit (cat Bio-52086-BIOLINE) allows convenient processing of multiple samples in 20 minutes. A critical requirement is that the DNA must be free of contaminants such as hemoglobin, polysaccharides, alcohol or high salt concentrations, as these may interfere with the PCR.Blood collection tubes coated with anticoagulants may inhibit the PCR, However these inhibitors will be eliminated by the use of the isolation kits given above. It is recommended to avoid the usage of heparin blood.

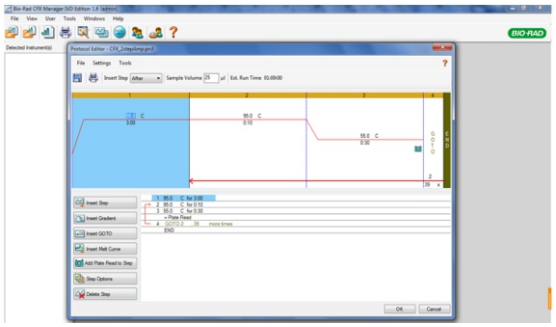
Quantitation

The quantitation standards provided in the kit (HIV RNA Standard 1-5) are treated in the same way as extracted samples and the same volume is used i.e. (15µl) instead of the sample. To generate a standard curve in the CFX96™ -IVD (BIORAD). Pipette 10 µl of the Master Mix into each labelled PCR tube. Then add 15 µl of the earlier extracted RNA to each sample tube and mix well by pipeting up and down. Correspondingly, 15 µl of the Standards (HIV S1-5) must be used as a positive control and 15 µl of water (Water, PCR grade) as a negative control. Close the PCR tubes and transfer the same into CFX96™.

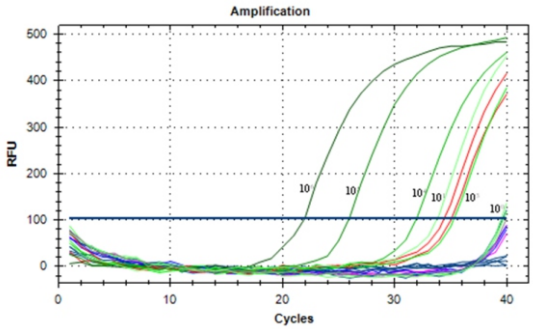
Programming the CFX96™.

Setting of general assay parameters & Reaction volume.

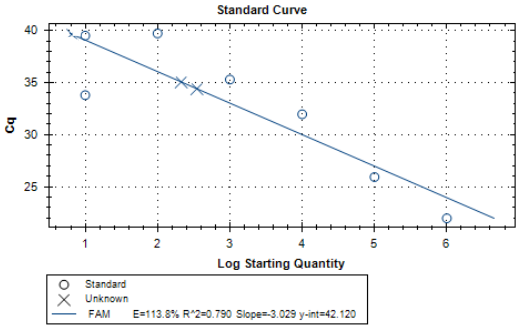
Bio-rad CFX Manager IVD edition 1.6 is an user friendly software. The parameters can be set in the protocol setting in just 3-4 step. On a single click the software will automatically go to the next function.



Example Figure 1: Showing Protocol setting of Bio-rad CFX Manager IVD edition 1.6



Example Figure 2: Graphical representation of real-time PCR data on patient sample: Indicating amplification of HIV LTR in all HIV-1 DNA Standard (106-100) as well as unknown sample . RFU is plotted against cycle. The threshold (Blue line) is set. The Ct value of target recorded. Amplifications were performed using the Bio-rad CFX Manager IVD edition 1.6.



Example Figure 3: Variation of Ct with PCR efficiency. The blue standard curve has an efficiency of 113% (the slope is –3.029).