หอสมุดกลาง สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

LITERATURE REVIEW

Human Immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) is the causative agents of AIDS (Acquired Immunodeficiency Syndrome). In 1983, there were two reports of recovery of a virus containing reverse transcriptase enzyme (RT) from lymph node of a man with persistent lymphadenopathy syndrome (LAS) by Montagnier *et al* and the isolation of HTLV from individuals with AIDS by Gallo *et a.l*(15, 16) Later in 1983, Montagnier and colleagues showed that the isolated virus they isolated, which was later called LAV (lymphodenopathy-associated virus), (17) could productively replicate in CD4+ lymphocyte. In 1984, Gallo and colleagues characterized HTLV-III, which was isolated from peripheral blood mononuclear cell (PBMCs) of AIDS patients(18). Also, Levy and colleagues reported a retroviruses named the AIDS-associated retroviruses (ARV) isolated from AIDS patients in 1984(19). The three prototype viruses (LAV, HTLV-III, and ARV) were recognized as members of the same group of retroviruses in Lentiviridae family.

HIV-1, HIV-2 are member genus Lentivirinae of family Retroviridae. They are enveloped plus-strand RNA viruses, with a diameter of about 110 nm. Infectious particles contain two identical copies of single-strand RNA of about 9 – 10 kilobases. These are surrounded by structural proteins that form the nucleocapsid and the matrix shell, to which a lipid envelope derived from the host cell membrane are inserted in this envelope. One feature that distinguishes the lentiviruses from other retroviruses is the remarked complexity of their viral genomes. Most retroviruses that are capable of replication contain only three structural genes- namely, *gag*, *pol*, and *env*(20). The *gag* and *env* genes encode the core nucleocapsid polypeptides and surface-coat proteins of the virus, respectively, whereas the *pol* gene gives rise to the viral reverse transcriptase and other essential enzymes. There are six additional regulatory genes- *vif*, *vpu*, *vpr*, *tat*, *rev*, and *nef*. It is the distinct but concerted action of these additional genes that probably underlie the profound pathogenicity of HIV-1.

A group of related viruses called simian immunodeficiency viruses (SIV) naturally infect various species of Old World monkeys and the chimpanzees. These primate lentiviruses are categorized into five major lineages. Lineage 1 contains various isolates of HIV-1 which are subclassified into three group, namely M (main), O (outlier), and N(21). The extraordinary variability of HIV, due to rapid mutation and recombination, has led to the development and geographical distribution of various distinctive clades, or subtypes, of viruses(7, 22). HIV-1 group M is divided into subtypes A to K. Isolates of group O differ as much from each other as do viruses from different subtypes of groups M, but their limited number has so far precluded a definition of distinct subtypes. Group N viruses have been isolated from only two individuals from Cameroon(23). Five subtypes of HIV-1 (A to E) have been defined. HIV-1 subtype B viruses were responsible for the first epidemic in North America, Europe, Australia, and the metropolitan center of other continents in the late 1970s and early 1980s. Subtype F has been found in Brazil and Romania, and G is prevalent in Russia. Almost all HIV-1 subtypes are found in Africa, with subtypes A, C, and D being the most prevalent in that continent. Subtype C is also rapidly spreaded worldwide and most globally prevalent subtype,(24) whereas group O viruses are rarely isolated and almost exclusively restricted to person originating from Cameroon, Gabon, and Guinea. Three major HIV-1 subtypes are dominant in Asia (C, CRF_01AE and B). Subtype C is the most common from in India. In Southeast Asian countries such as Thailand, Cambodia, Burma, and Vietnam, the circulating recombinant from: CRF01_AE dominates, although subtype B and C are fairly common. Subtype B predominates in Japan, Taiwan, and the Philippines, although CRF_01AE has recently become more prevalent in the Philippines. In China, diverse HIV-1 strains have been identified. Subtype B, C, CRF01_AE, and CRF08_BC are circulating in individuals who acquired HIV infection sexually, whereas subtypes CRF08_BC (more than 80%) and CRF07_BC have been detected in injecting drug users. But in the HIV epidemic in paid blood donors in Henan and Hubei provinces, HIV-1 subtype B is most common. Active recombination is underway in the region, and new forms of HIV-1 intersubtype C/B and C/B /E recombinants with different recombination breakpoints have recently been reported from central Burma and from China(24).

The transmission of HIV usually requires transfer of bodily fluids. The most important of these are blood, semen, and vaginal secretions that contain the virus, or may permit the transfer of cells, especially macrophages, containing virus(25). In general, HIV is transmitted between humans in there ways(26).

1. Sexual transmission: sexual contact with an infected person
    - male to male
    - male to female
    - female to male

2. Blood-borne transmission: exposure to infected blood or blood component from an HIV-infected donor
    - Blood transfusion
    - Shared injection equipment

3. Vertical transmission
    - perinatally from an infected mother to her child
    - postpartum from nursing mother (presumably through breast feeding)

Sexual transmission accounts for the majority (75%) of cases of HIV Infection worldwide. The number unprotected sexual contacts, the stage of infection (which may dictate the viral load) and the existence of genital ulceration may all increase the risk of transmission and thus play important role in the sexual spread of HIV.

Infection typically begins when an HIV particle encounters a cell with a surface molecule called cluster designation 4 (CD4). Cells with this molecule are known as CD4 positive (CD4+) cells. One or more of the virus gp 120 molecules binds tightly to CD4 molecule(s) on the cell surface(27, 28). The membrane of the virus and cell fuse, process that probably involves both gp41 and a second "fusion cofactor" molecule on the cell surface, known as chemokine receptor,(29, 30) although CD4+ T cells appear to be the main target of HIV, other immune system cells with CD4 molecules on their surface are infected as well. These include monocytes, macrophages, Langerhans cells of the skin, follicular dendritic cells in the lymph node, alveolar macrophages in the lung, retinal cells, and cells of the uterine cervix. In addition, HIV may infect microgial cells in the brain

which may not bear CD4 surface proteins(31-33). Cell-to-cell spread of HIV also can occur through the CD4-mediated fusion of an infection cell with an uninfected cell. Following fusion, the HIV RNA, protein, and enzymes are released into the target cell.

After attachment and internalization of HIV, the HIV virion is partially uncoated in preparation for replication phase of its life cycle(34). Viral replication in cytoplasmic compartment retains nucleocapsid proteins with the generation of a complementary DNA (cDNA) copy of the viral RNA which is mediated by the reverse transcriptase (RT) enzyme encoded by HIV. Six of the nine antiretroviral drugs approved in the United States for the treatment of people with HIV infection (AZT, ddC, ddI, d4T, 3TC and nevirapine) work by interfering with this stage of the viral life cycle(35). Second-strand DNA synthesis is also controlled by the reverse transcriptase but proceeds only after the action of a second *pol* gene product, ribonuclease H which partially degrades the original RNA template. The newly made HIV DNA moves to the cell nucleus, where it is spliced into the host DNA with the help of HIV intergrase. Once incorporated into the cell genome, HIV DNA is called a "provirus"(36, 37).

For a provirus to produce new viruses, the special LTR sequence contains the appropriate promoter, enhancer, and other signals required for transcription of genes by the host RNA polymerase II(36, 38). Some of the RNA will be used to form the genetic material of new viral particles and some will be used to direct the translation of HIV structural proteins or regulatory proteins, which work to control viral replication. The new virions are produced from multiple copies of the viral proteins. These proteins are form as large precursors - long chain protein molecules, which are then specifically cleaved to become the enzymes and structural proteins of the new virions.

The assembly of a new virus particle begins with two of precursor proteins collecting at the edge of the cell, where they join together and attach themselves to the host cell membrane. They begin to form a spherical structure, which bulges outwards from the cell membrane and draws two strands of viral. RNA into it. An enzyme called the final steps of protein cleavage as follows: first, it cuts itself free from the polyprotein molecule; then, the protease works to cleave all the other viral components from the cycle. Eight drugs (Indinavir, Ritonavir, Saquinavir, Nelfinavir, Lopinavir/Ritonavir, Atazanavir, Tipranavir/Ritonavir) have been approved(2). The remaining protein segments make up the

protein coat that surrounds the RNA and the viral enzymes, forming the inner 'capsid', at the core of the virus particle. A third structural protein, the envelope glycoproteins, which together with elements from the host cell membrane, totally enclose the new virus particle, which leaves the cell in process known as budding(35).

The Immunopathogenesis of HIV Infection

The rate of progression of HIV disease may be substantially different among HIV-infected individuals. Following infection of the host with any virus, the delicated balance between virus replication and immune response to the virus determines both the outcome of the infection, i.e. the persistence versus elimination of the virus and different rates of disease progression(39, 40).

i) Clinical Course of HIV Infection.

The clinical course of HIV infection generally includes three phases or stages-primary infection, clinical latency and AIDS. Such a course of infection is characteristic of the so-call typical progressors when represent the majority of HIV-infection individuals. The natural history of HIV in Asia varies from what has been seen in other parts of the world. In Asia, progression from HIV infection to AIDS is faster than in the West: 6.9 years in a Thai cohort and 7.9 years in an Indian study, compared with 10 years or more in most pre-HAART western cohorts(24).

ii) Primary Infection

Approximately three to six weeks after initial infection, 50-70% of HIV-infected individuals develop an acute mononucleosis-like syndrome. This period is associated with high levels of viremia, and within one week to three mounts there is an antibodies response to HIV. This immunity is apparently inadequate to suppress viral replication completely, since HIV expression persists in lymph nodes even when plasma viremia is difficult to detect. Detectable viremia declines markedly or disappears weeks to mounts after the acute syndrome subsides. Although a substantial percentage of patients with HIV infection do not have a clinically recognizable acute syndrome after primary infection, the events described above probably occur even in the absence of symptoms.

iii) Clinical Latency

Most patients have a period of "clinical latently" that lasts for years after primary infection, viral distribution, the appearance of HIV-specific immunity, and the apparent curtailment of viral replication. During this period virtually all patients have a gradual deterioration of their immune system, manifested particularly by the depletion of CD4+ T cells. Although this depletion may occur even without large increases in plasma concentrations of virus (as manifested by p24 antigenemia, viral RNA levels or culturable virus), viral replication in lymphoid organs, together with the spectrum of immunologic events that are directly or indirectly triggered by the virus, may contribute to it. Thus, HIV disease is clearly progressive during the so-called latent period.

iv) AIDS-Defining Illness

AIDS-defining illness or clinically apparent disease is the inevitable outcome of the progressive deterioration of the immune system that occurs in most patients with HIV infection. Exceptions to the direct correlation between deteriorating immune function and clinically apparent disease are the progressive generalized lymphadenopathy; Kaposi's sarcoma, which can occur before the onset of sever immunosuppression; and neurologic disease that may reflect direct or indirect effects of the virus or its products on neurons. The profound immunosuppression that occurs during this phase of HIV infection is the end stage of the immunopatogenic events that began at the time of primary infection, and continued for years through the clinically latent but microbiologically active stages of infection.

v) Typical Progressors

The majority (70-80%) of HIV-infected individuals belong to the group of typical progressors. Following primary infection, as mentioned above, typical progressors experience a long period (up to six to eight years) of clinical latency. Despite the lack of symptoms, HIV disease is active as is indicated by the persistent replication of virus and by the progressive loss of CD4+ T-cells. Individuals with CD4+ T-cells counts > 500 per cumm[3] generally remain free of symptoms, whereas the appearance of constitutional symptoms is generally more frequent in individuals with CD4+ T-cell counts below 500

per $cumm^3$. Exceptions to this paradigm are subjects with CD4+ T-cell counts higher than 500 per $cumm^3$ who progressive generalized lymphoadenopathy, Kaposi's sarcoma, or neurologic diseases. Progression to clinically apparent disease or AIDS-defining illness generally occurs within eight to ten years in typical progressors. Then CD4+ t-cell counts are below 200 per cumm3, the clinical picture may be characterized by severe and persistent constitutional signs and symptoms; at this level of CD4+ T-cells, there is an increased susceptibility to opportunistic infections or neoplasms.

vi) Rapid Progressors

A significant percentage (10-12%) of HIV-infected individuals experience an unusually rapid progression to AIDS with two three years of primary infection. Rapid progressors may experience a prolong acute viral syndrome and the period of true clinical latency may be absent or very brief. Downregulation of the initial burst of viremia may not be very efficient in rapid progressors; even after the initial decrease, the levels of viremia may rise rapidly. Inefficient control of the initial burst of viremia and rapid rise in viremia within the first or second yeas after primary infection reflect a poor control of HIV infection by the immune system. In this regard, a delay in the appearance of the primary immune response or a rapid disappearance if certain immune functions during the early stages of the chronic phase of infection may be detected in rapid progressors.

vii) Long-Term Nonprogressors

A small percentage (less than 5 % on the basis of different cohorts) of HIV-infected individuals does not experience progression of disease for an extended period of time. Long-term nonprogressors by some definitions have CD4+ T-cell counts that are within the normal range are stable over time; in addition, they generally have low levels of virologic parameters and preservation of lymphoid tissue architecture and immune function. From a clinical standpoint, long-term nonprogressors are asymptomatic; it seems that in these individuals, HIV infection has been arrested with regard to disease progression. It is unknown whether long-term nonprogressors have experienced a primary infection similar to that of other groups of HIV-infected individuals, i.e. associated with an acute viral syndrome and burst of viremia.

viii) Long-Term Survivors

In a small percentage of subjects who experience progression of HIV disease within a period of time similar to typical progressors, both clinical and laboratory parameters, although abnormal, remain stable for an extended period of time. The mechanisms, either virology or immunologic, that are responsible for preventing further progression of HIV disease are unclear at present; the possibility that changes in virus genotype and/or phenotype, as well as the possibility that preservation of certain HIV-specific immune response are involved, is being investigated.

The average time between infection and development of AIDS is about 11 years, but approximately 20% of infected individuals progress rapidly to AIDS within five years. Another 12% of infected individuals remain free of AIDS for up to 20 years. Viral replication in lymphocytes, approximately 10 billion or so virus particles a day, is associated with the defeat of immune system. HIV kills the cells, which is why high level of virus in blood causes advance immunosuppression and AIDS. This defeat of the immune associated with low CD4 T-cell levels. This defeat of the immune system is associated with the development of conditions like cytomegalovirus infection of the retina, *Pneumocystis carinii* infection of the lung, or tumors like Kaposi's sarcoma or non Hodgkin's lymphoma.

Untreated human immunodeficiency virus type 1 (HIV-1) infection is characterized by an early acute phase with high levels of viremia that leads to a clinically asymptomatic phase of variable duration, followed by immunodeficiency(41). Throughout infection an extraordinarily large number of viral replication cycles occurs, and this high replicative capacity of HIV-1 leads to both varied genetic pools and high viral loads(42). The absolute level of steady-state plasma viremia (viral load) is a strong predictor of the rate of disease progression and in combination with CD4 T cells counts, has great prognostic value. The base-line HIV-1 RNA concentrations are highly predictive of prognosis. These is a strong time dependent prognosis relation between HIV-1 RNA and out come and reduce concentrations of HIV-1 RNA, in response to antiretroviral therapy, are predictive of improved prognosis(7). Plasma viral load is widely used to monitor antiretroviral drug therapy and is more valuable as a marker of antiviral drug efficacy than changes in CD4 T cell counts(43-45).

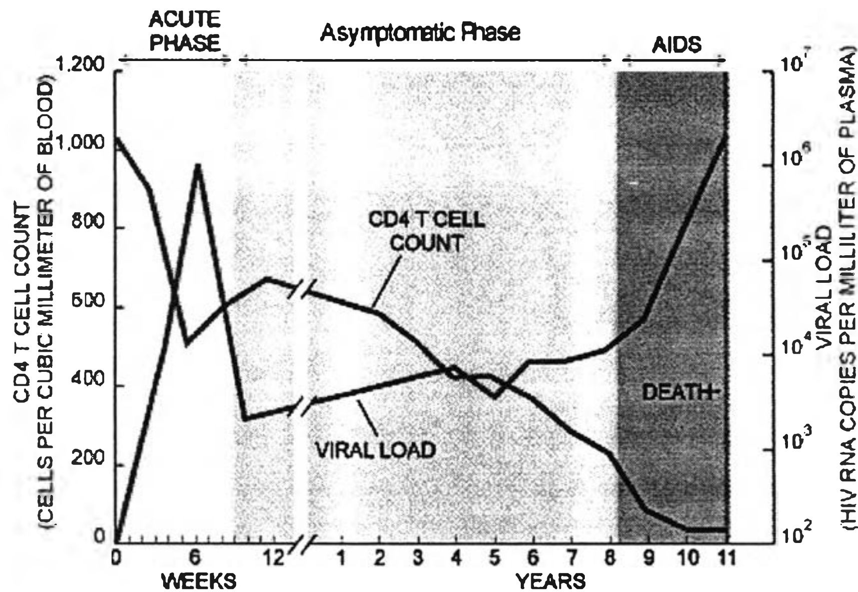

Figure 1 Hypothetical course of HIV infection in adults

As HIV infection progresses, the viral load level again begins to rise (see Figure I). Studies have recently indicated that a high level of HIV-1 RNA in the plasma at baseline is the most powerful predictor of progression to AIDS. A study using the Chiron bDNA assay showed that patients with a baseline of >100,000 eq/mL were 10-11 times more likely to progress to AIDS within five years(46). Likewise, studies using the Roche Monitor assay showed that individuals with a baseline of >250,000 copies/ml had a frequency of disease progression of 60 percent or greater(47). In addition to predicting risk of disease progression, viral load testing may be useful in predicting risk of transmission. Studies have indicated that mothers with higher levels of HIV RNA in plasma are more likely to transmit the virus to their infants(48, 49). In the era of antiretroviral therapy, plasma HIV-1 RNA level or viral load is one of the most imports monitoring laboratory to assess the response to the treatment or indicated by the level is below 50 copies/ml.

Important lessons have been learned in Thailand about providing national level access to antiretroviral programmes for people living with HIV/AIDS. Several prerequisites and actions are needed in parallel to ensure efficient access to HAART: (i) committed leadership at the policy level; (ii) significant reductions in ARV costs; (iii) strengthening of the health care system and infrastructure; (iv) expanded ability of care providers to manage HIV care through training to improve attitudes and build knowledge and skill in treatment of patients with HAART; (v) reduced cost for treatment monitoring, especially CD4+ counts and viral loads (vi) comprehensive patient education on the

principles of HAART, adherence, and its toxicity; (vii) involvement of the community (non-profit organizations and people living with HIV/AIDS groups) in the process; (viii) locally relevant clinical research—eg, HIV-NAT at the TRC-ARC has been doing many clinical trials, which not only contribute to region-specific HIV care, but provide HAART access for over 1500 patients; and (ix) gaining supplemental international funding support—eg, from the Global Fund for AIDS, Tuberculosis and Malaria.(24)

For this reason, there are many approaches and assays available for quantifying HIV-1 RNA. The three most widely used commercial assays are described here, with attention to aspects of each assay that may favor its use in different clinical situations.

The HIV-1 Quantiplex (bDNA) Assay

The Quantiplex HIV-1 RNA assay (Chiron Diagnostics, Norwood, MA) is a branched DNA (bDNA) sandwich method which quantifies plasma HIV-1 RNA by amplifying the signal rather than the target nucleic acid.[50-52] The bDNA assay dose not require viral RNA purification or PCR amplification steps. Instead, virion are concentrated by high speed centrifugation and disrupted by detergent and proteinase K to release viral RNA. This lysate is the incubated with two sets of oligonucleotides. The first set captures viral RNA, hybridizing to both conserved regions of the HIV-1 *pol* gene and to oligonucleotides bound to microwell. The second set of oligonucleotides provides signal amplification. This set consists of four components: oligonucleotides with homology to both the target RNA and to preamplifier oligonucleotides; and oligonucleotide probes bound to alkaline phosphatase (AP). Each of these components hybridized to at the multiple sites. In this way, the signal is amplified without copying the target RNA. Detection is accomplished by chemiluminescence using an AP-specific substrate. The amount of light detected is directly proportional to the amount of bound nucleic acid. The absolute quantity of HIV-1 RNA is determined from an external standard curve being run on the same plate.

An important advantage of signal amplification is the avoidance of quantification errors inherent in extraction and amplification of the target nucleotide sequence. In comparative studies with assays based on target amplification, the reproducibility of the Quantiplex HIV-1 RNA assay is often superior,(5, 53-55)especially in the low end of

dynamic range. In addition, plasma substances and anticoagulants such as heparin that may impair RT-PCR do not adversely affect the bDNA assay. The bDNA assay oligonucleotide probes bind equally to HIV-1 group M subtypes A-F allowing viral load measurements of diverse HIV-1 strains. The disadvantages of the bDNA assay are the absence of an internal quantitation standard for each sample and the requirement of a relatively large sample volume (at least 1 ml of plasma) which may pose a problem when sample volume is limited, such as in pediatric specimens.

The AMPLICOR HIV-1 MONITOR Assay

The AMPLICOR HIV-1 MONITOR assay (RT-PCR, Roche Diagnostic, Corporation, Branchbura, NJ) is the only FDA- approved HIV-1 RNA quantification assay. It is based on reverse transcription (RT) of the target HIV-1 RNA and polymerase chain reaction (PCR) amplification of the resulting cDNA. Viral RNA is extracted from either acid citrate dextrose (ACD) or EDTA anticoagulated plasma with guanidine isothiocyanate. Nucleic acid from the relatively impure lysate is precipitated with isopropanol. Reverse transcription and PCR amplification occur in a single step using the thermostable recombinant enzyme *Thermus thermophilus* DNA polymerase (*rTth pol*) which has both RT and DNA polymerase activity. Segments of the viral cDNA are amplified exponentially to a high copy number (amplicon) with repeated cycles of heating and cooling in the presence of biotinylated oligonucleotide primer specific to conserved region of HIV-1 gag, *rTth pol*, deoxynucleotide triphosphates, and appropriate buffer components. Amplicons are denatured and single-stranded DNA is bound to microwells coated with HIV-1 specific oligonucleotide probes. An avidin-horseradish peroxidase (HRP) conjugate is added to bind to the biotin-labeled amplicon. The amount of avidin-HRP bound amplicon is determined after the addition of an HRP-specific colorimetric substrate.

The AMPLICOR HIV-1 MONITOR assay uses an internal quantification standard at a known concentration that is added to each sample before extraction, both to quantify the sample HIV-1 RNA and to compensate for plasma inhibitory factors affecting extraction and amplification. The quantification standard consists of RNA transcribed *in vitro* that is identical in size to the target amplicon and uses the same HIV-1 gag primer binding sites, generating an amplicon that is also captured on the microtiter well. A

colorimetric readout occurs through an enzyme-linked detection system, differentiating the internal standard and target amplicons.

The NucliSens HIV-1 Assay

The NucliSens HIV-1 QT assay is based on target amplification using NASBA (Nucleic Acid Sequence Based Amplification) technology (Organon Tekniika, Duram, NC). The NASBA assay selectively and directly amplifies HIV-1 RNA without PCR in a one step sandwich hybridization procedure using two oligonucleotide primers, three enzymes, nucleoside triphosphates and appropriate buffer.[34] First, highly purified RNA is extracted using guanidine thiocyanate and silicon dioxide particles. The RNA is amplified by repeated cycles of synthesis and transcription of a double-stranded DNA intermediate. An oligonucleotide primer (P1) specific to a region in HIV-1 gag is used to synthesize cDNA from the specimen RNA template using Avian Myeoblastosis Virus (AMV) reverse transcriptase. The RNA strand is degraded by RNase H, allowing the oligonucleotide primer P2 to bind and initiate second-strand DNA synthesis. Anti-sense RNA is then transcribed off the double-stranded DNA via T7 polymerase promoter (originally incorporated by P1). This cycle is repeated resulting in exponential amplification (1 million to 1 billion-fold) under isothermal conditions. The amount of nucleic acid is determined directly by chemiluminescence, which is characterized by very high sensitivity and a broad dynamic range. Quantitation of HIV-1 viral load is accomplished by co-amplification of three internal RNA quantitation standards specific for HIV-1 *gag* and part of *pol.*

The advantages of HIV-1 quantification by the NucliSense HIV-1 QT assay are that relatively small plasma sample volume is used (0.1 ml is standard, although the assay can accommodate a wide range of sample volumes (0.01-2.0 ml), and there is a large dynamic range (80-1,000,000 RNA copies/ml).(56) The reaction is rapid (90 minutes), takes place in a single tube, and does not require thermal cycle. In addition, the extracted RNA product is relatively pure and free of interfering substances that inhibit PCR amplification, such as those in heparinized blood plasma or seminal plasma(57). For this reason, the NucliSens HIV-1 QT assay is commonly used for determining viral load in many types of tissues in which interfering substances may be present.

Despite the reduction in cast of anti-HIV drug, however, the cost of HIV viral load test still remains expensive. This brings up the research objective to develop the method to measure HIV RNA load by in house method. Real-time PCR application in most setting

and prompting for quantitation the development of reliable cost-effective of HIV-1 RNA in plasma compare with commercial kit and in-house assays(10).

Performance characteristics of assay for HIV viral load

Sensitivity: The assay sensitivity is defined as lowest level of HIV RNA that can be detected 95% of the time. This statistical method of assessing sensitivity is generally considered to be the standard to determine the lower quantitative "limit of detection (LoD)" for quantitative HIV RNA assay. There are currently four FDA-approved assays for measuring HIV-1 viral load: Bayer VERSANT ® HIV-1 RNA 3.0 Assay (bDNA), Roche Amplicor HIV-1 MONITOR® Test version 1.0 and 1.5 (PCR), and the bio Merieux Nuclisens ™ HIV-1 QI Test. According to package insert, the sensitivity claim of the Bayer bDNA is 75 copies/ml, the Roche PCR assay is 50 copies/ml, and the bio Merieux NASBA assay is 176 copies/ml(58). Clinically, the goal of therapy is to suppress viral load to below the LoD.

Accuracy: The assay accuracy is refers to the ability of the assay to determine the true value of an analyze. Vary difficult to establish "gold" standard for viral load (VL). Calibration of assays to common standard should result in consistency of quantification between assays.

Precision or reproducibility: the reproducibility of an assay is defined by capacity to achieve very similar viral quantification values regardless of the laboratory, day operator, and instrument or kit lot. Determines the change in viral load that is statistically significant.

Specificity: High specificity means that HIV-negative samples will not be positive in a viral load test for HIV.

Linearity: the linearity is referring to which the assay standard curve approximates a straight line. Used to determine the linear range of the assay. Linearity is a measure of how accurately the assay measures changes in viral load throughout its dynamic rang.

Dynamic range: the dynamic range of an assay is defined as the quantitative over which the assay can reliable report result. Since viral levels are obtained at varying time points during treatment regimens, it is essential that the assay be able to accurately follow change in HIV RNA across the wide range of clinically expected values. The bDNA methodology utilized a single assay to span the entire dynamic range of 75 to 500,000 copies/ml. By comparison, the PCR methodology used two separate assays fined that an

ultrasensitive version that has a dynamic range of 50 to 750,000 copies/ml and a standard version that has an over lapping dynamic rang of 400 to 750,000 copies/ml.

Principle of "real-time" PCR

The application of fluorescent techniques to the RT-PCR, together with suitable instrumentation capable of combining implication, detection and quantification, has led to the development of kinetic RT-PCR methodologies that are revolutionizing the possibilities for quantitative nucleic acids(59). Two important findings led to the discovery of real-time PCR. First, the finding that the Taq polymerase possesses a 5' ⟶ 3' exonuclease activity(60). Second, the construction of dual-labeled oligonucleotide probe, which only emits a fluorescent signal upon cleavage of the probe, based on the FRET (Fluorescence Resonance Energy Transfer) principle. There are four competing instruments on market; all four are run as closed-tube systems and quantification requires no post-amplification manipulation . This avoids problems of data acquisition and analysis and minimizes hands-on time. The entire process, starting at RT and ending with full quantification, is automated, which makes these instruments ideally suit high-throughput screening amplification(61).

The ABI prism 7700 (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA) contains a built-in thermal cycler with 96-well positions, and is able to detect fluorescence between 500 nm and 660 nm. Fluorescence is induced during the RT-PCR by distributing laser light to all 96 samples contain in thin-walled reaction tubes via a multiplexed array of optical fibers. The resulting fluorescent emission returns via the fibers and is directed to a spectrograph with a charge-coupled device (CCD) camera. Because each well is irradiated sequence can be used for spectral resolution of the fluorescent light. This instrument can be used for assays based on DNA-binding dyes, molecular beacons and hybridization probes. RT-PCR reactions typically take 2 hours to complete. Its description by the manufacturer as a real-time technique is not quite correct as, unlike completing systems, the progress of the RT-PCR reaction can be analyzed only after the amplification completes.

The computer software constructs amplification plots using the fluorescent emission data that are collected during the PCR amplification. The ΔRn values are plotted versus the cycle number. The measurements from cycle 3 until 15 are considered as the baseline. During the early cycles of the PCR amplification, the ΔRn stays in the baseline.

An arbitrary threshold is chosen based on the variability of the baseline, usually determined as 10 x the SD of the baseline being set from cycle 3 to 15. This can be manually changed for each individual experiment if necessary. Threshold cycle (Ct) values are then calculated by determining the point at which the fluorescence exceeds this chosen threshold limit. Ct is reported as the cycle number at this point. Therefore, Ct values decrease linearly with increasing input target quantity. This can be used as a quantitative measurement of the input target.

The high specificity of this method is due to a complementary between the set of primers, the internal probe and the target. The fluorescent signal will only be generated if the probe is annealed to the target sequence during the PCR amplification.

The Lightcycler (Roche Molecular Biochemicals, Bachringer Mannheim, Germany) performs the RT-PCR in small-volume glass capillary tubes contain within a rotor-like carousel that are heated and cooled in an airstream(62). The carousel is rotated past a blue light-emitting diode, and fluorescent is read by three photodetection diodes with different wavelength filters that allow the use of spectrally distinct fluorescence probes(63). The performance of the Lightcycler in terms of sensitivity and specificity is equal to that of Taqman(64). In addition to being able to use assays based on DNA-binding dyes, hydrolysis probe and molecular beacons, it can be used for dual hybridization probes. Up to 32 reactions are typically carried out in 5-20 μl volumes(65) and RT-PCR reaction is completed in less than 20 min(66). The Lightcycler is a truly real-time technique, and the fluorescence readings taken at every cycle of the PCR reaction are displayed immediately after each measurement, allowing amplification runs to be terminated or extended as appropriate during individual runs.

The iCycler iQ from Biorad Instruments is another option for the real-time PCR. It is an optical module that can be connected in the thermal cycler and reads the fluorescence during the PCR exponential phase. The real-time analysis can be viewed during measurement. According to the manufacturer's specifications, the iCycler is able to multiplex four different fluophores per sample tube. Moreover, 96 samples can be tracked simultaneously that provides a much quicker assay.

A new option is the Mx4000™ Multiplex from Stratagene. The manufacturer claims that their sequence detector is able to detect multiple fluorescent PCR chemistries, including TaqMan probes, single dye primers, molecular beacons, etc. The design of the sample block accommodates samples in a variety of formats (96-well plates, 8-strips tubes, or individual tubes). The light source for the Mx4000 system is a quartz tungsten halogen lamp that generates a broad excitation range of 350 to 750nm. The system generates real time amplification plots that can be viewed as a PCR run progress.

Techniques for real-time PCR

There are currently five competing techniques available for detection of amplified product with the same sensitivity(67). They use fluorescent dyes and combine the progresses of amplification and detection of an RNA target to permit the monitoring of PCR reactions in real-time during the PCR. Their sensitivity eliminates the need for second-round amplification and decreases opportunities for generating false-positive results(68). The simplest method uses fluorescent dyes that bind specifically to double-stranded-DNA. The other four rely on the hybridization of fluorescence-labeled probes to the correct amplicons. These methods differ in their specificity, although at later amplification cycles all can show artifacts that do not correlate with specific product accumulation. As amplicon detection in the molecular beacon, hydrolysis, hybridization and scorpion probe assays depend on successful obviate the need for post-PCR Southern analysis or sequencing to confirm the identity of the amplicon. Currently there is a range of different possibilities in chemistries available for real-time PCR. They are:

DNA-binding dyes

This method involves detection of the binding of a fluorescent dye (SYBR Green) to DNA(69). The unbound dye exhibits little florescence in solution, but during elongation increasing amounts of dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerization step, and falls off when the DNA is denatured. Consequently, fluorescence measurement at the end of elongation step in every PCR cycle are performed to monitor the increasing amount of amplified DNA. This method obviates the need for target-specific florescent probe, but its specificity is determined entirely by its primers. As

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the presence of any double-stranded DNA generates fluorescence, this assay is not more specific than conventional RT-PCR. However, additional specificity and RT-PCR product verification can be achieved by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon(70). This is done by slowly increasing the temperature above the T_m of the amplicon and measuring the fluorescence. As the T_m of the amplicon depend on markedly on its nucleotide composition, it is possible to identify the signal obtain from the correct product. Once the melting curves are established you can set the software to acquire fluorescence above the primer dimers'melting temperature but below that of the product. Also a careful optimization of the reaction can reduce the formation of primer dimers to a level that is only important for very low copy detection.

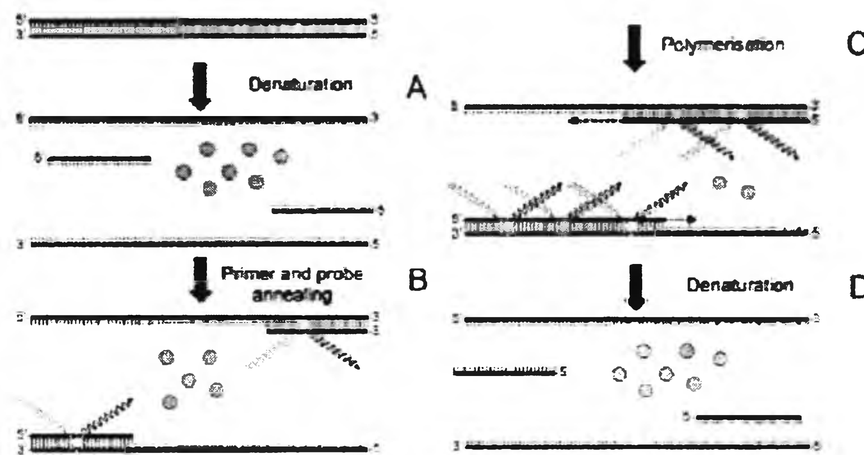

Figure 2 DNA-binding dyes assay

Hydrolysis probes

The Taqman assay (Perkin-Elmer-Applied Biosystems) utilizes the 5'-nuclease activity of the DNA polymerase to hydrolyse a hybridization probe bound to its target amplicon. It usually utilizes either *Taq* or *Tth* polymerase but any enzyme with an equivalent 5'-nuclease activity properties (e.g. *Tfl*) can be used(71). After the reverse transcription step, successful quantification requires the annealing of three oligonucleotides to the DNA. Two template-specific primers define the endpoints of the amplicon and provide the first level of specificity. The additional specificity of this assay is provided by the use of a third oligonucleotide probe that hybridizes to the amplicon during the

annealing/extension phase of the PCR. The probe contains a fluorescent reporter dye at its 5' end such as FAM (6-carboxyfluorescein), or a TET (tetrachloro-6-carboxyfluorescein), JOE(2,7,-dimethoxy-4,5-dichloro-6-carboxyfluorescein), HEX (hexacholoro-6-carboxyfluorescein) and VIC, the emission spectrum of which is quenched by a second fluorescent dye at its 3' end such as TAMRA (6-carboxytetramethylrhodamine). If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound. As the 5'-exonucleasee activity of *Taq* and *Tth* polymerase is double-strand-specific(72), unbound probes remain intact and no reporter fluorescence is detected. Conversely, if the correct amplicon has been amplified, the probe can hybridize to that amplicon after the denaturation step. It remains hybridized while the polymerase extends the primers until it reaches the hybridized probe. When it displaces its 5' end to hold it in a forked structure, the enzyme continues to move from the now free end to the bifurcation of duplex, where cleavage takes place(73). This separates the reporter and quencher dyes and releases quenching of reporter fluorescence emission(74). The largest fluorescence signal is obtained when the two labels are at the extreme 5' and 3' of probe, probably because of more efficient cleavage by the polymerase(75). The increase in fluorescence is measured cycle by cycle and is a direct consequence of the amplification process. As the polymerase will cleave the probe only while it remains hybridized to its complementary strand, the temperature conditions of the polymerization phase of the PCR must be adjusted to ensure probe binding. Most probes have a T_m of around 70 °C; therefore, the Taqman system uses a combined annealing and polymerization step at 60-62 °C. This ensures that the probe remains bound to its target during the primer extension step. It also ensures maximum 5'-3' exonuclease activity of the *Taq* and *Tth* DNA polymerase.

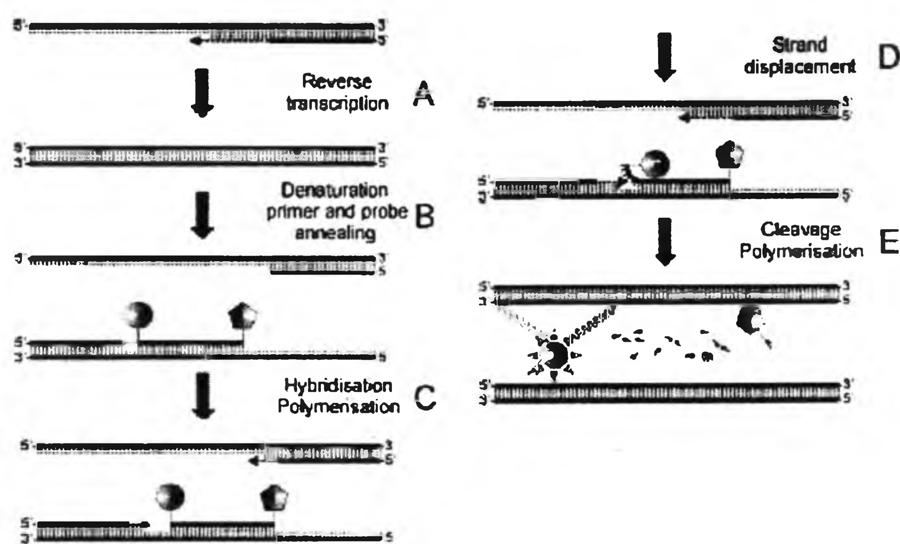

Figure 3 TaqMan probe assay

Hybridization probes

This method uses two hybridization probes to maximize specificity. One of the probes carries at its 3’ end a fluorescein donor, which emits green fluorescent light when excited by the Lightcycler’s light source. Its emission spectrum overlaps the excitation spectrum of an acceptor fluorophore that is attached to the 5’ end of the second probe. This probe must be blocked at its 3’ end to prevent its extension during the annealing step. Excitation of the donor results in fluorescence resonance energy transfer to the acceptor and the emission of red fluorescent light. In solution, the two dyes are apart, and because the energy transfer depends on the spacing between two dye molecules and only background fluorescence is emitted by the donor. After denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. This brings the two dyes in close proximity to one another and the fluorescein can transfer its energy at high efficiency. The intensity of the light of longer wavelength emitted by the second dye is measured with increasing amounts of measured fluorescence proportional to the amount of DNA synthesized during the PCR reaction. A fluorescent signal is detected only as results of two independent probes hybridize to their correct target sequence. This increases specificity and generates additional flexibility for probe design. Furthermore, as

the probes are not hydrolyzed, fluorescence is reversible and allows the generation of melting curves.

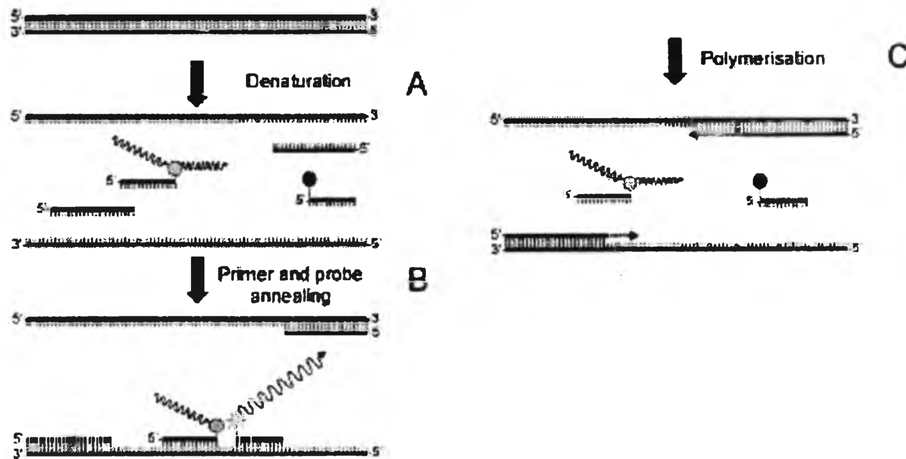

Figure 4 Hybridization assay

Molecular beacon

Molecular beacon (Stratagene) is DNA hybridization probe that form a stem-and-loop structure; the loop portion of the molecule is complementary to the target nucleic acid molecule and the stem is formed by the annealing of complementary arm sequence on the ends of the probe sequence(76). A fluorescent marker is attached to the end of one arm and a quencher is attached to the end of the other arm. The quencher is a non-fluorescent chromospheres that dissipates the energy that it receives from the fluorophore as heat. In solution, free molecular beacon adopt a hairpin structure and the stem keeps the arms in close proximity, resulting in efficient quenching of the flurophore. When molecular beacon encounter a complementary target at the annealing temperature, they undergo a conformational transition that forces the stem apart and results in the formation of a probe/target hybrid that is longer and more stable than the stem(77). This separates the fluorophore and the quencher, leading to the restoration of fluorescence which can be detected. Whereas any free molecular beacon remains close and non-fluorescence. If the target DNA sequence dose not exactly matches the molecular sequence, hybridization and fluorescence will not occur. This is because the thermodynamic properties of the molecular beacon favors the formation of a hairpin rather than continue to hybridize to a less than

perfectly matched target sequence.

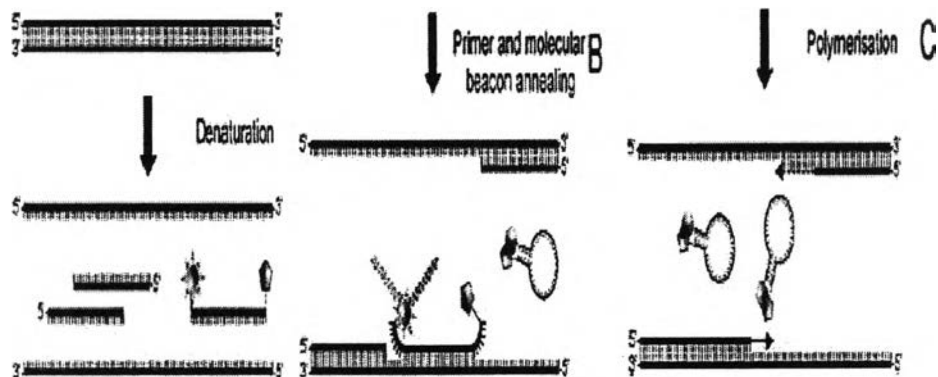

Figure 5 The molecular beacons assay.

Scorpion probes

A Scorpion consists of a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequence on either end. A fluorophore is attached to the 5' end giving a fluorescent signal that is quenched in the hairpin loop configuration by a moiety joined to the 3'end. The hairpin loop is linked to the 5' end of a primer. After extension of the Scorpion primer, during amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed. A PCR stopper between the primer and the stem sequence prevents read-through of the hairpin loop, which could lead to the opening of the hairpin loop in the absence of the specific target sequence. The unimolecular nature of the hybridization event gives rise to significant advantages over homogeneous probe systems. Unlike Molecular Beacon and Double-Dye Oligonucleotides assays (for which Scorpions can be used as an alternative technology), Scorpion assays do not require a separate probe.

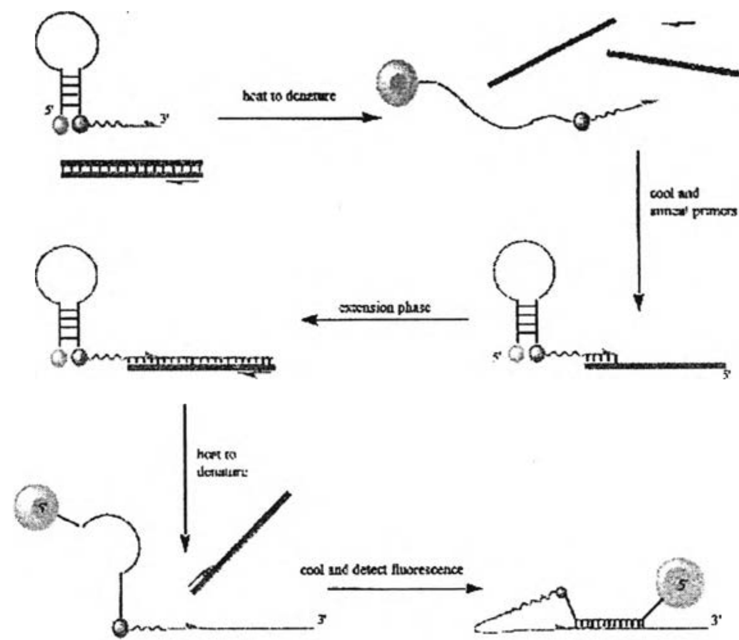

Figure 6 Scorpion probe assay

For quantitated HIV RNA viral load, RNA cannot serve as a template for PCR. The first step in an RT PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. Usually, this involves the use of dedicated RNA- and DNA-dependent DNA polymerase in separate (two-enzyme/two tube) reaction. Separation of RT and PCR step has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely but high risk of contamination and uses long time to calculate. Alternatively, a single polymerase able to function both as an RNA- and DNA-dependent DNA polymerase can be used in an one enzyme/one tube reaction (one-step RT-PCR).

The concentration of HIV-1 RNA in the plasma of HIV infected individuals is an important predictor of disease outcome and a marker of antiretroviral drug efficacy.[6] Of the Food and Drug Administration (FDA)-approved tests for HIV-1 RNA in plasma, the most sensitive have detection limits of 50 copies/ml(78) and treatment is generally considered initially successful if the level of viremia can be reduced to below this level. However, reduction of the HIV-1 RNA load to 50 copies/ml does not guarantee long-term success, and a rebound of drug resistance can occur(79). More sensitive assays for HIV-1 RNA are needed to detect, quantify, and characterize persistent viremia in patients who are

receiving antiretroviral therapy and whose plasma HIV-1 RNA levels are suppressed to less than 50 to 75 copies/ml(41).

More recently, Real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma was developed. The efficacy of the assay was determined initially using serial dilutions of the HIV-2 NIHZ standard and TaqMan system. The Ct-value was determined in six independent assays of serial dilutions of the HIV-2 NIHZ standard made in diethyl pyrocarbonate (DEPC)-treated water. The sensitive of the assay was 50% at 50 virus particles/ml input and 100% at all higher input samples (10^2-10^6 virus particles/ml). The detection limit of the assay was therefore set at 10^2 virus particles/ml, although samples with 50 virus particles/ml are detected regularly at Ct-values above 40. The linear range of the assay was at least 4 logs with a Spearman regression coefficient of 0.99 and the inter-assay variability based on the dilution series Ct-value ranged from 2% at 106 virus particles/ml to 7.5%at the detection limit (10^2 virus particles/ml). the intra-assay variability was determined by processing 32 samples of 104 particles/ml and proved to be 2.5%(80).

In 2002, real-time quantitative PCR method for measuring the HIV-2 RNA was developed on LightCycle system. The stock of HIV-2 strain NIHZ, which was counted by electron microscopy, was as the standard. Several primer sets targeting the highly conserved *gag* region were evaluated. The sensitivity of assay was 100%at a viral load of 250 copies/ml and 66% at a viral load of 125 copies/ml. There are correlation between the CD^4+-cell count, the clinical stage, and the plasma RNA level. The last factor seems to be a good indicator of clinical status, making it the marker of choice for prospective follow-up and monitoring of treatment. In contrast, while the proviral DNA level correlates with the CD^4+-cell, it does not correlate with the clinical status(81).

In 2003, the quantification of HIV-1 RNA concentrations down to 1 copy/ml of plasma was developed on ABI 7700 Sequence Detection System (Applied Biosystems). This assay with single-copy sensitivity (the single-copy assay) generates a reproducible linear regression plot of input copy number versus threshold cycle by using HIV-1 RNA transcripts at copy numbers ranging from 1 to 10^6 per reaction mixture. The single-copy assay was compared to the Ultrasensitive AMPLICOR HIV-1 MONITOR assay and a more sensitive modification of the ultrasensitive assay by repeatedly testing a low-copy-

number panel containing 200 to 0.781 copies of HIV-1 RNA per ml of plasma. This comparison showed that the single-copy assay had a greater sensitivity than the other assays and was the only assay that detected HIV-1 RNA at levels as low as 0.781 copies/ml. Testing of plasma samples from 15 patients who were receiving antiretroviral therapy and who had <75 HIV-1 RNA copies/ml revealed persistent viremia in all 15 patients, with HIV-1 RNA levels ranging from 1 to 32 copies/ml (median, 13 copies/ml). The greater sensitivity of the single-copy assay should allow better characterization of persistent viremia in patients who are receiving antiretroviral therapy and whose HIV-1 RNA levels are suppressed to below the detection limits of present assays, although sensitivity is maximum with 7 ml of plasma(41).

In 2004, quantitative detection of HIV-1 viral load by SYBR green real-time RT-PCR technique in HIV-1 seropositive patients was developed, the result demonstrated that this technique detected 50 HIV-1 RNA copies /ml of plasma. Moreover, Comparison of real-time RT-PCR with the b-DNA technique considered widely a reference technique for HIV-1 RNA viral load measurement. The parallel quantitative analysis of HIV-1 positive samples showed a high correlation (r =0.908) between the two methods. Although b-DNA and the real-time-based method gave similar sensitivity, the assay determined quantitatively HIV-1 RNA copies in 4 out of 16 samples shown as undetectable by b-DNA. The SYBR green real-time RT-PCR represents a good alternative to b-DNA assay in HIV-1 viral load determination especially during the monitoring of HAART treatment(12).

We have optimized and clinically evaluated a simplified in-house real time RT-PCR system. The goal of the study is to reduce the risk of contamination which may occur during the transferring step, to shorten the time used for analysis and more importantly to lower the overall cost.