CHAPTER II

LITERATURE REVIEWS

1. Global and Thailand HIV/AIDS epidemic

The total number of people living with the human immunodeficiency virus (HIV) rose in 2004 to reach its highest level ever: an estimated 39.4 million (35.9 million–44.3 million) people are living with the virus. These include the 4.9 million (4.3 million–6.4 million) people who acquired HIV in 2004. The global AIDS epidemic killed 3.1 million (2.8 million–3.5 million) people in the past year.

The number of people living with HIV has been rising in every region, compared with two years ago, with the steepest increases occurring in East Asia, and in Eastern Europe and Central Asia. The number of people living with HIV in East Asia rose by almost 50% between 2002 and 2004, an increase that is attributable largely to China's swiftly growing epidemic. In Eastern Europe and Central Asia, there were 40% more people living with HIV in 2004 than in 2002. Accounting for much of that trend is Ukraine's resurgent epidemic and the ever-growing number of people living with HIV in the Russian Federation. Sub-Saharan Africa remains by far the worst-affected region, with 25.4 million (23.4 million–28.4 million) people living with HIV at the end of 2004, compared to 24.4 million (22.5 million–27.3 million) in 2002. Just under two thirds (64%) of all people living with HIV are in sub-Saharan Africa, as are more than three quarters (76%) of all women living with HIV. The epidemics in sub-Saharan Africa appear to be stabilizing generally, with HIV prevalence at around 7.4% for the entire region. But such a summary perspective hides important aspects. First, roughly stable HIV prevalence means more or less equal numbers of people are being newly infected with HIV and are dying of AIDS. Beneath the apparent constancy of steady prevalence levels lie devastating realities especially in southern Africa, which accounts for one third of all AIDS deaths globally. Second, the epidemics in Africa are diverse, both in terms of their scale and

the pace at which they are evolving. There is no single "African" epidemic. Some urban parts of East Africa display modest declines in HIV prevalence among pregnant women, while in West and Central Africa prevalence levels have stayed roughly steady at lower levels than in the rest of sub-Saharan Africa. National HIV data, though, hide much higher levels of infection in parts of countries, as Nigeria illustrates. Southern Africa, unfortunately, offers only slight hints of possible future declines in HIV prevalence. HIV prevalence in the Caribbean is the second-highest in the world, exceeding 2% in five countries, and AIDS has become the leading cause of death among adults aged 15–44 years in this region. Yet, a growing number of Caribbean countries are showing that the epidemic does yield to appropriate and resolute responses.

Thailand, the first case of AIDS was found in 1984 but the documented AIDS case returned home for terminal care. In February 1985, three patients were clinically diagnosed before the commercial HIV-1 test kit was available(12). The first explosive spreading of HIV occurred among injection drug users (IDU's) in Bankok about 1 % in 1987 to 43% in 1988(13). In the later year, the trend of increasing of HIV infection was found in the commercial sex workers (CSW's) in the upper north region of Thailand, and reached 63% in 1991(14).

2. HIV-1 Terminology and structure

2.1 HIV-1 terminogy

HIV-1 belongs to the *Lentivirus* subfamily of retroviruses and has been shown to be the etiologic agent of acquired immunodeficiency syndrome (AIDS) (15, 16). It was genetically divided into two groups: M (major group) and O (outlier group). Group M is pandemic worldwide and contains at least 10 subtypes. The major subtype in Thailand is subtype E while the B is the major subtype in USA and Europe. Recently, new group termed N (non-M-non-O) has now been identified from Cameroon (17).

2.2 Structure and genome organization of HIV-1

HIV-1 is spherical in shape and has a diameter of 100 to 120 nm. The outer coat of the virus, known as the viral envelope, is a lipid bilayer that taken from the membrane of the host cell when a nascent virion buds from the infected cell. The envelope contains several cellular membrane proteins, including major histocompatibility antigens (MHC) and viral proteins. The proteins that protrude from the envelope surface consists of gp120 (the surface protein, or SU) and gp41 (the transmembrane protein, or TM). The gp120 contains the binding site for the CD4 receptor, and the seven transmembrane domain chemokine receptors that serve as co-receptors for HIV-1. The gp41 contains the outer N-terminal hydrophobic domain which initiates the process of virus-cell membrane fusion, the transmembrane and inner cytoplasmic tail that anchor to the lipid bilayer. The inner surface of the envelope lining with matrix proteins (MA, p17) to which it is attached by covalently bound myristic acid. Within the envelope of a mature HIV-1 particle is a cone-shaped capsid, consists of 2,000 copies of major capsid proteins (CA), p24. The capsid surrounds two copies of single strands RNA genome, nucleocapsid protein (NC), p7/p9, three viral enzymes: protease, reverse transcriptase and integrase and the viral accessory proteins: Nef, Vif and Vpr. The structure of HIV-1 is shown in Figure 1A and 1B.

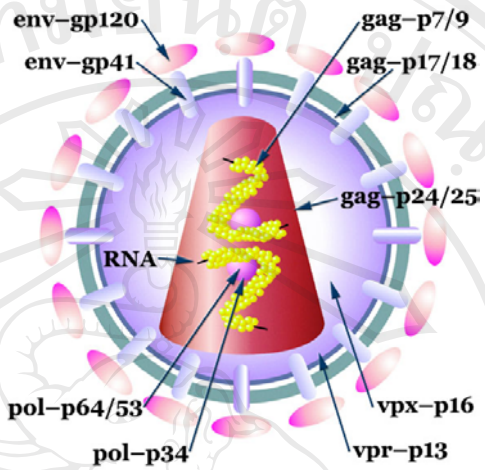

Figure 1A The structure of HIV-1 particle

http://www.aids.harvard.edu/research/discoveries.html [online avialable (Nov 1,2005)]

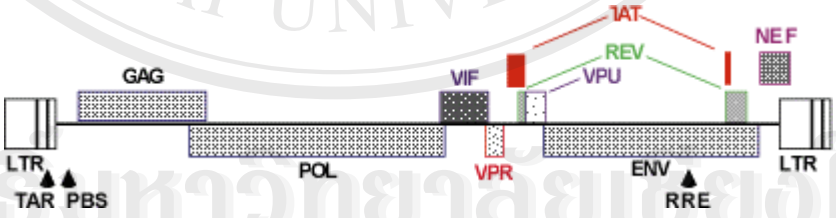

Figure 1B Diagram of HIV-1 genomic RNA

http://www.bioscience.org/1999/v4/d/macphers/fulltext.htm [online avialable (Nov 1,2005)]

3. Infection and pathogenesis

3.1 The infectious cycle of HIV-1

3.1.1 Binding and entry

The infection of cells by HIV-1 begins with the interaction of the viral envelope proteins with two cell surface proteins, CD_4 and HIV-1 co-receptor. The CD_4 is an extracellular immunoglobulin (Ig)-like structure containing 4 domains and is expressed on the surface of a subset of T-lymphocytes and some macrophages. The gp120 of Env bound to the CDR2-like region in the first extracellular Ig-like domain of CD_4. Binding of the gp120 to CD_4 induces conformational changes in the gp120 that create or expose a binding site for a co-receptor. The association of gp120 with co-receptor drives additional conformational changes within the entire trimeric gp120/gp41 complex and eventually led to the fusion between the viral envelope and the host cell membrane.

3.1.2 Reverse transcription and DNA synthesis

Subsequent to internalization and uncoating, the HIV-1 genomic RNA was reverse transcribed to cDNA and then double-stranded proviral DNA using viral Pol proteins containing the RT and ribonuclease H enzymatic activities (RT/RNase H). The reverse transcription process also generates the LTR regions on the both 5' and 3' ends of the proviral DNA that are characteristic of retroviruses and necessary for integration into the cellular chromosomal DNA.

3.1.3 Nuclear transport of the pre-integration complex

The double-stranded viral DNA pre-integration complex contains the Gag matrix, Pol integrase-RT proteins and the nuclear localization signals that may pivotal in targeting the nucleoprotein complex to the nucleus. In this step, the pre-integration complex has to transverse an intact nuclear membrane for access to the host chromosomal DNA.

3.1.4 Proviral DNA integration

The linear double-stranded viral DNA is capable of integrating into the host chromosomal DNA with the help of HIV-1 integrase that contained in the pre-integration complex. Once incorporated into the host chromosomal DNA, HIV-1 DNA is called a provirus. The proviral DNA can persist for many years in a latent state and secretly carrying the genetic instructions for making new virions.

3.1.5 Viral transcription and protein synthesis

When the host cell is activated, the proviral DNA can be transcribed by the host cell polymerases into viral mRNA, and consequently translated into the viral proteins. Together, the viral genomic RNA and proteins then migrate to the host cell membrane, where they assembled to new virions.

3.1.6 Assembly and budding of virus

Before the virion can be released from the cell, the viral proteins must coordinately assemble. The viral enzyme and genomic RNA gather just inside the cell membrane, while the viral envelope proteins aggregate within the membrane. An immature viral particle forms and pinches off from the cell, acquiring an envelope that includes both cellular and HIV-1 proteins from the cell membrane.

During this step, the core of the virus is still immature and the virus is not yet infectious. The long chains of proteins and enzymes that make up the immature viral core are now cleaved into smaller pieces by a viral enzyme called protease. This step results in infectious viral particles.

3.2 Pathogenesis of HIV-1 infection

3.2.1 Primary HIV infection

Primary infection general refers to the period from initial infection until the immune response to HIV gain some measure of control over viral replication, usually a few weeks to months. Approximately 30 to 70 % of newly infected individual experience signs and symptom during acute HIV infection that may include fever, maaise, rash, lymphadenopathy, pharyngitis, headache,

diarrhea and occasionally neurologic manifestations(18) This complex is referred to as the acute retroviral syndrome.

Primary HIV infection is characterized by active viral replication and extremely high level of plasma viremia(4). During this period, the virus disseminated thought out the body and seeds lymphoid organs, where its replication is incompletely suppressed(19). Concomitant with the high level of viremia is the frequent observation of a precipitous decline in CD_4+ T-cell counts. The initially high level of HIV replication and plasma viremia generally decrease with the appearance of an HIV-specific immune response. The decreasing viral levels gradually stabilize with in 6 months to 1 year at a virologic "set-point". During primary infection, the number of cytotoxic T lymphocyte (CTL) precursors directed against the viral Gag, pol, and envelope proteins correlates with a decrease in the burst of plasma viremia. The cellular immune response appear to be more important than the humoral response in controlling HIV replication during acute infection, because neutralizing antibodies against HIV are not detected for at least 30 to 60 days after the resolution of the peak viremia (20). CD_8+ T-cell-mediated soluble, suppressor like activity has been reported in some patients during primary HIV infection. This non-lytic activity also appears to be temporally associated with a decrease in plasma viremia, a finding suggesting that it, too, may play an important role in controlling viral replication (21).

3.2.2 Clinical latency

This phase is characterized by chronic immune activation and persistence viral replication despite a lack of consistent signs or symptom of disease. Typically, this is the longest lasting of the three stages of HIV infection, representing approximately 80% of the total course (18). During this phase, the number of circulating CD_4+ T cells slowly declines by about 50 to 70 cells/μL per year, signaling the onset of progressive immune deficiency. The declining CD_4+ T-cell numbers are likely go to ongoing viral replication with accompanying direct cytotoxicity, immune-mediated elimination of infected cells, and failure to replace adequately the dying CD_4+ T cells. The pivotal role of CTLs in

controlling virus replication continues throughout the chronic phase of infection. However, the specificity of the CTLs, as well as the magnitude of the response, varies of among infected individual (22). Neutralizing antibodies are also present throughout the asymptomatic phase of disease, albeit at relatively low level (23, 24). In addition to neutralizing antibodies, binding antibodies to Env, Nef, Rev, Vpr, Vpu, and Tat are also detected, but they tend to decline as disease progresses. Antibodies to p24 rise to their highest levels during this phase and then fall, usually undetectable levels, before the onset of AIDS.

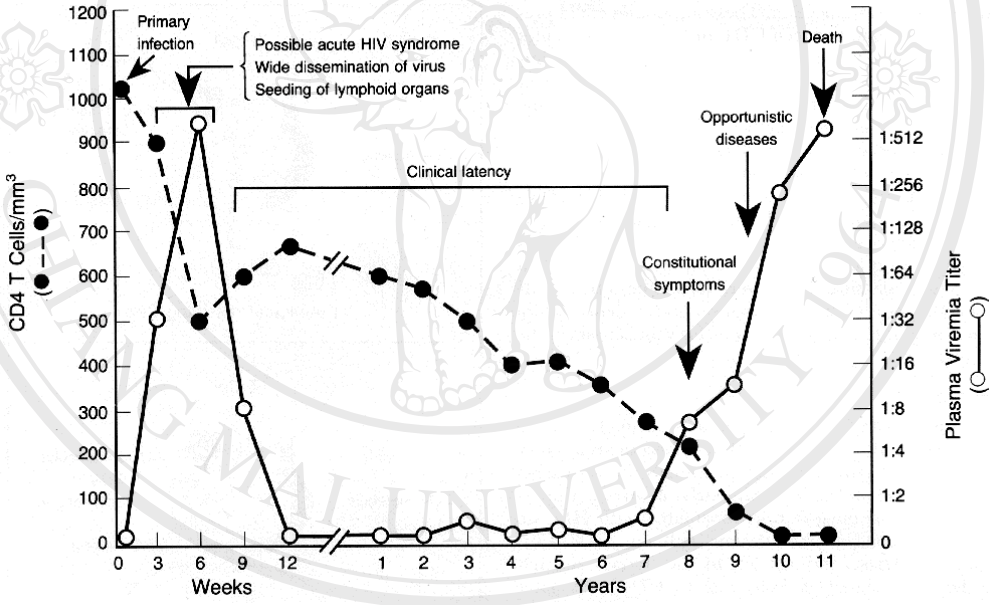

Figure 2 Typical course of HIV infection
(Adapted from Pantaleo G, Graziosi C, Fruci AS. The immunopathogenesis of human immunodeficiency virus infection. N Engl Med 1993; 328:327-335.)

3.2.3 Advance disease

Advance disease is characterized by an AIDS-defining illness or a decline in the levels of circulating CD_4+ T cells to below 200 cells/μL(25). Plasma viremia usually increases during stage of disease(18) and is correlated with a sharp decrease in CD_4+ T-cell counts. Immune function is diminished, as evidenced by the loss of CD_4+ T cells, the destruction of lymphoid tissue

architecture, and the loss of CD_8+, CTLs and noncytolytic viral suppression. Levels of neutralizing antibodies are generally low or undetectable at this stage of disease, and titers of non-neutralizing antibodies to viral proteins are diminished (22). Finally, the late stage of disease is also characterized by destruction of lymphoid tissue and collapse of the follicle dendritic cells (FDCs) network.

4. Antiretroviral drugs

As the late 2001, 16 antiretroviral drugs had been approved for use in the United States and several more were in various stage of clinical development. These drugs are all direct against two targets, reverse transcriptase and protease, with the inhibitor of the former being classified as nucleosides or non-nucleosides.

The use of these drugs in the management of HIV-infected patients has developed into remarkably complex specialty. These drugs must be used in various combinations of three or more drugs, addressing the principle of viral dynamic, pathogenesis, and drug resistance. Treatment success is related to the rigorous adherence to talking multiple pills usually at least twice daily.

New antiretroviral drugs designed against new targets should not be affected by resistance in protease and RT. Moreover, they may have unique attributes regarding both antiviral activity and toxicity. Potent compound have been reported against three additional targets in HIV.

Inhibitors of HIV fusion have been identified that target either the virus surface glycoprotein or the host cell cytokine receptors by the viruses (26). T-20 and T-1249 are peptides corresponding to an extracellular domain of the transmembrane segment (gp41) of HIV envelope glycoprotein. The documentation of 2 $\log_{10}$ unit reductions in plasma HIV RNA level after 14 days of treatment with T-20 established that fusion is an appropriate antiviral target with clinical potential, prompting the search for better inhibitors of this target.

Table 1 Approved antiretroviral drugs

Drugs	Major toxicities
Nucleoside RT inhibitors	
Zidovudine (AZT)	Anemia, neutropenia, nausea, headache, insomnia,myalgia and weakness, elevated serum lactate level, steatohepatitis, fat redistribution
Zalcitabine (ddC)	Peripheral neuropathy
Didanosine (ddI)	Peripheral neuropathy, pancreatitis, sicca syndrome
Stavudine (d4T)	Peripheral neuropathy, elevated liver function test, elevated serum lactate, steatohepatitis, fat redistribution
Lamivudine (3TC)	
Abacavir (ABC)	Nausea, anorexia, abdominal pain, hypersensitivity reaction
Tenofovia	-
Nonnucleoside RT inhibitors	
Nevirapine	Rash, hepatitis
Efavirenz	Rash, CNS symtomes (abnormal concentration and dreams, dizziness
Delavirdine	Rash, increase liver function tests
Protease inhibitors	
Saquinavir	Diarrhea,abdominal pain , nausea
Ritonavir	Circumoral parathesia, altered test, diarrhea, weakness, nausea
Indinavir	Nausea, abdominal discomfort,nephrolithiasis,dry skin, nail change, indirect hyperbilirubinemia
Nefinavia	Diarrhea, nausea, weakness, abdominal pain
Amprenavir	Rash, nausea, diarrhea
Lopinavir	Diarrhea, fatique, nausea

5. Monitoring CD4 and viral load

The measurement of plasma HIV RNA concentrations provides a sensitive indicator of the risk of disease progression and death in patients with asymptomatic HIV infection. The ability to measure plasma HIV-1 RNA concentrations may permit a more targeted, rational use of antiretroviral drugs before the onset of severe immunodeficiency. The risk of disease progression among the mostly asymptomatic subjects enrolled in this study was better indicated by the plasma HIV-1 RNA concentration than by the CD_4 cell count, which was not as strong a predictor of clinical events(14, 27).

Many studies have demonstrated the correlation of plasma HIV-1 RNA levels with stage of disease. For patients with high viral load titer are progresses more rapidly than lower RNA viral titer. Such as, individuals with plasma HIV-1 RNA level >100,000 copies/mL with six months of seroconversion are 10-time more likely progress to AIDS within five years than patients with lower level of plasma (28).

6. Viral load assays

In most cases, untreated HIV-1 infection is characterized by high-level viral production and CD_4 T cell destruction progressing despite an often lengthy clinical latency period to significant net loss of CD_4 T cells and AIDS(29, 30). The absolute level of steady-state plasma viremia ("viral load") is a strong predictor of the rate of disease progression, and, in combination with CD_4 T cell counts, has great prognostic value (3, 28, 31). Plasma viral load measurements are widely used to monitor changes in viremia during antiretroviral drug therapy, and are more valuable as a marker of antiviral drug efficacy than changes in CD_4 T cell counts (3, 27, 28, 32).

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.

6.1 Reverse transcriptase polymerase chain reaction (33)

The AMPLICOR HIV-1 MONITOR® Test amplifies and detects a 142 base target sequence located in a highly conserved region of the HIV-1 *gag* gene, defined

by the primers SK431 and SK462. The *gag* region encodes the group specific antigens or core structural proteins of the viron. The HIV-1 gag genes are generally about 1,500 nucleotides in length and are located at the approximate positions 789-2,290 in the HIV genome. The reaction is performed with thermostable recombinant enzyme *Thermus thermophilus* DNA polymerase (r*Tth* pol). In the presence of manganese and under the appropriate buffer condition, r*Tth* pol has both reverse transcriptase (RT) and DNA polymerase activity. This allows both reverse transcription and PCR amplification to occur in the same reaction mixture.

6.1.1 Reverse Transcription

The processed specimens are added to the amplification mixture in reaction tubes in which both reverse transcription and PCR amplification occurs. The downstream or antisense primer (SK431) and the upstream or sense primer (SK462) are biotinylated at the 5' ends. The reaction mixture is heated to allow the downstream primer to anneal specifically to the HIV-1 and QS target RNA. In the presence of excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and thymidine triphosphates, r*Tth* pol extends the annealed primer forming a complementary (cDNA) strand.

6.1.2 PCR Amplification

Following reverse transcription of the HIV-1 and QS target RNA, the reaction mixture is heated to denature the RNA:cDNA hybrid and expose the HIV-1 and QS target sequences. As the mixture cools, the upstream primer anneals to the cDNA strand, the r*Tth* pol catalyzes the extension reaction, and a second DNA strand is synthesized. This completes the first cycle of PCR yielding a double stranded DNA copy (or amplicon) of each HIV-1 or QS RNA. The reaction mixture is heated again to separate the resulting double stranded DNA and expose the primer target sequences. As the mixture cools, the primers anneal to the target DNA. r*Tth* pol, in the presence of excess dNTPs, extends the annealed primers along the target templated to produce a 142 base primer pair sequence termed an amplicon.

Amplification occurs only in the region of the HIV-1 genome between the primers. The entire HIV-1 genome is not amplified.

6.1.3 Selective Amplification

Selective amplification of target nucleic acid from the clinical specimen in the Amplicor HIV-1 Monitor® Test is achieved by the use of AmpErase and deoxyuridine triphosphate (dUTP). AmpErase (uracil-N-glycosylase, UNG) recognizes and catalyzes the destruction of DNA strands containing deoxyuridine, but not DNA containing thymdine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contain deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase prior to amplification of the target DNA. AmpErase, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine containing DNA at deoxyuridine residues by opening the deoxyribose chain at the c1 position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase is inactive at temperatures above 55C; i.e. throughtout the thermal cycling steps and therefore does not destroy target amplicon. Following amplification, any residual enzyme is denatured by the addition of the denaturation solution, thereby preventing the degradation of target amplicon. AmpErase in the Amplicor HIV-1 Monitor® Test has been demonstrated to inactivate 10 copies of deoxyuridine-containing HIV-1 amplicon per PCR.

6.1.4 Hybridization reaction

Following PCR amplification, the HIV-1 and QS amplicons are chemically denatured to form single stranded DNA by the addition of denaturation solution, and aliquots are added to separate wells of a microwell plate coated with HIV-1 specific (SK102) abd QS-specific (CP35) oligonucleotide probes. HIV-1 and QS amplicons are bound to HIV-1 and QS wells, respectively, by hybridization to the mircorwell plate bound probes. To achieve quantitative results over a large dynamic, range, serial dilutions of the denatured amplicon are analyzed in the microwell.

6.1.5 Detection reaction

Following the hybridization reaction, the microwell plate is washed to remove unbound material and an avidin-horseradish peroxidase conjugate (Av-HRP) is added to each well of the plate. The AV-HRP binds to the biotin labelled amplicon captured by the plate bound oligonucleotide probes. The plate is washed again to remove unbound AV-HRP and a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenxidine (TMB) is added to the wells. In the presence of hydrogen peroxide, the bound horseradish peroxidase catalyzes the oxidation of TMB to form color compexes. The reaction is stopped by addition of a weak acid, and the optical density at 450nm is measured using an automated microwell plate reader.

6.1.6 HIV-1 RNA Quantitation

The Amplicor HIV-1 Monitor® Test quantitates viral load by utilizing a second target sequence (QS) that is added to the amplification mixture at a known concentration. The QS is a non-infectious 219 base pair in vitro transcribed RNA molecule with primer binding region identical to those of the HIV-1 target sequence. The QS, therefore, contains SK431 and SK462 primer binding sites and generates a product of the same length (142 bases) and base compostion as the HIV-1 target. The probe binding region of the QS was modified to differentiate QS-specific amplicon from HIV-1 target amplicon.

The optical density in each well of the plate is proportional to the amount of HIV-1 or QS amplicon in the well, and the total optical density is proportional to the amount of HIV-1 or QS RNA, respectively, input into each reverse transcription/PCR amplification reaction. The amount HIV-1 RNA in each specimen is calculated from the ration of the total optical density for the HIV-1 specific well to the total optical density for the QS-specific well and the input number of QS RNA molecules using the following equation:

$$\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}} \times \text{Input QS copies per PCR reaction} \times 40 = \text{HIV-1 RNA copies/ml}$$

6.2 bDNA signal amplification (Quantiplex™ HIV-1 RNA)(6)

The branched DNA technique is a signal amplification assay. In the first step, target nucleic acids are immobilized by hybridization to capture probes on a solid phase. Several different species of oligonucleotides, which act as bridges to branched nucleic acids, are then hybridized at multiple sites to the target nucleic acid. Finally, over 700 alkaline phosphatase molecules can attach to each immobilized target nucleic acid. Detection relies on enzymatic reaction with the chemiluminescence's substrate. Because no amplification products are generated, contamination is unlikely. Within 24 hours, 42 tests can be run.

6.3 NASBA(34)

The NASBA technology has been use for the detection of primary RNA targets. This technique generates RNA amplicons with polarity opposite that of the input RNA. No applification of background DNA will occur using this technology. The detection of this RNA amplicon can be done in real time with molecular beacons. This method is an isothermal-based RNA amplification technique, which employs three different enzymes active at 41ºC: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase. Two primers are necessary, including a forward primer that contains a T7 RNA polymerase promoter sequence. Following amplification, a double-stranded DNA amplimer is formed that contains this T7 RNA polymerase promoter sequence. From this template, thousands of RNA transcripts are synthesized with polarity opposite that of the origin target sequence. Recently, molecular beacons have been used as a detection and quantification system for NASBA to monitor the amplification of human immunodeficiency virus (HIV) type 1 isolate form genotypes M, N and O(35).

7. Comparison of the commercial HIV-1 RNA viral load assays

Several studies of comparation of three commercially quantitative HIV-1 RNA assays were shown in Table 2 The high correlation was approved.(36, 37). At present, all available commercial viral load assays have the lowest limit detection at

50 copies/ml by Ultrasensitive Amplicor HIV-1 Monotor® 1.5 assay (approximately 50-80 copies/ml)(Table 2.)(36, 38, 39). Serial testing of clinically stable patients not on antiretroviral therapy or on a stable falling regimen has shown the relative stability of plasma. HIV-1 RNA levels over the short term, with a biological variation approximately 0.3-0.4 log10 copies/ml (40, 41). So, Changes of the plasma HIV-1 RNA greater than 0.5-0.7 log10 (3-5 fold) are likely to reflect significant changes in HIV-1 replication(42).

8. Real-time polymerase chain reaction

Real-time PCR is a real-time or on-line analysis of PCR products which produced during amplification in a close system without requirement of post manipulation of PCR processing.

In conventional PCR, quantification either requires multiple samples or aliquots must be taken from a single sampler at certain intervals. The amplification product then has to be detected by gel electrophoresis and ethidium bromide staining or Southern blotting. These procedures have several disadvantages. Preparation of multiple samples is expensive, withdrawal of aliquots very often lead to contamination, agarose gel analysis lacks of sensitivity and specificity and Southern blotting has vary laborious. The accuracy of these techniques is vary limited no matter which method is used. By contrast, the real-time PCR employ the fluorescent detection system to measure the amount of the PCR products at each and every cycle (9). The product yield is plotted against the cycle number. The result is an amplification plot, a curve that represents the accumulation of product over the duration of the entire PCR reaction. Tracking the entire reaction allow quantification of PCR product during the exponential phase of the reaction, when reagents are not depleted and reaction kinetics favor doubling of the PCR product every cycle. Quantification of amplification product in the exponential phase, as opposed to the

Table 2 Characteristics of commercial assays for quantitation of plasma HIV-1 RNA

Assay	Range (copies/ml)	Intra-assay variation (log10 SD)	Inter-assay variation (log10 SD)
RT-PCR (HIV-1 Monitor 1.5)			
1. Standard	400-750,000	0.15-0.33	0.12-0.22
2. Ultrasensitive	50-75,000		
bDNA	50-500,000	0.13-0.23	0.05-0.17
NASBA	80-8,000,000	0.12-0.20	0.038-0.261

Note; Data were complied from reference number 42, 44, and 45

endpoint of the PCR reaction, underpins the precision of the real-time technique.

Current real-time PCR system can detected 10 or fewer copies of target, or as little as 0.1 pg of total RNA, within a two-fold precision and a dynamic range of six to eight orders of magnitude (34).

Real-time technology has a number of advantages. It is not surprising that diagnostic laboratories want and need to switch their labor-intensive quantification strategies to real-time technology. One of the bottlenecks in molecular diagnostics is that for most viral targets, laboratories have to develop their own in-house method (34). It is unlikely that commercial assays will be developed for those targets in the near future. Exceptions include such as viruses as HIV-1, HCV, and HBV, for which no real-time assay available. It is clear that there is a great deal of interest for clinically important viruses, for sample, those belonging to the Herpesviridae group (see table 3). Readymade reagents combined with several real-time reporter molecules and automate extraction methods warrant a more sophisticated approach. It is anticipated that viral targets for which virus culture is currently an alternative, will be routinely detected using these technologies.

Table 3 Human viruses detected with real-time amplification technology (34)

Virus	Detection technology
Herpes types 1 and 2	Hybridization probes
	TaqMan probe
Varizella zoster virus	Hybridization probes
	TaqMan probe
Epstein–Barr virus	TaqMan probe
Cytomegalovirus	Hybridization probes
	TaqMan probe
HHV6	TaqMan probe
HHV8	TaqMan probe
TTV	TaqMan probe
GBV	TaqMan probe
Polyomavirus	TaqMan probe
Hepatitis B virus	TaqMan probe
Hepatitis C virus	Hybridization probes
	TaqMan probe
Parvovirus B19	TaqMan probe
HIV type 1	NASBA—Molecular Beacon
	TaqMan probe
	PCR—Molecular beacon
HIV type 2	TaqMan probe
	Molecular beacons
HTLV 1 proviral DNA	TaqMan probe
Enterovirus	TaqMan probe
Influenza virus	TaqMan probe
Dengue virus	TaqMan probe
West Nile virus	TaqMan probe

8.1 DNA binding fluorophores

The basis of the sequence non-specific detection methods is the DNA binding fluorogenic molecule. Ethidium bromide (9), YO-PRO-1 (43, 44) and SYBR® green 1 (45) all fluorescence when associated with dsDNA which is expose to a suitable wavelength of light. This approach requires less specialist knowledge than the design of fluorogenic oligoprobes, is less expensive and does not suffer when the template sequence varies, which may abrogate hybridization of an oligoprobes (46). Formation of primer-dimer is common and, together with the formation of the PCR into the plateau phase(47, 48). Association of a DNA-binding dye with primer-dimer or other non-specific amplification products can confuse interpretation of the results. The problem of primer-dimer can also e addressed using solfware capable of fluorescenct melting curve analysis. This method makes use of temperature at which the dsDNA amplicon is denatured (T_D). The shorter primer-dimer can be discriminated by its

reduced T_D compared with the full-length amplicon. Analysis of the melting curve of the amplicon in the presence of SYBR green 1 has demonstrated that the practical sensitivity of DNA-binding fluorophores is limited by non specific amplification at low initial template concentrations.

DNA binding fluorophores also increase the T_D and broaden the melting transition, requiring substantial sequence change to produce a shift in the T_D. Oligoprobes are able to discriminate single point mutation using temperature at which 50% of oligoprobe-target duplexes separate(49). This temperature called the melting temperature (T_M) and it is depend upon the concentration of the dsDNA, its length, nucleotide sequence and the solvent composition(50).

8.2 Hybridization probe

The use of a pair of adjacent, fluorogenic hybridization oligoprobes was first described in the late 1980s(51) and, now known as "HybProbes", they have become the method of choice for the LightCycle™ (Roche Molecular Biochemicals, Germany), a capillary-based, microvolume fluorometer and thermocycler with rapid temperature control(52, 53). The upstream oligoprobe is labelled with a 3' donor fluorophore (FITC) and the downstream probe is commonly labelled with either a LighCycler Red 640 or Red 705 accepter fluorophore at the 5'terminus so that both oligoprobes are hybridized, the two fluorophores are located within 10 nt of each other. The plastic and glass composite capillaries are optically clear and act as cuvettes for fluorescence analysis, as well as facilating rapid heat transfer. Capillaries are rotated past a blue light-emitting diode and fluorescence is monitored by photodetection diodes with different wavelength filters. The temperature is varied by rapidly heating and cooling air using a heating element and fan which produce ramp rates of 20ºC/s, prolonging polymerase survival(45).

8.3 Hydrolysis probe (TaqMan probe)

In the late 1980s homogeneous assays were few and far between, but rapid advances in thermocycler instrumentation and the chemistry of nucleic acid manipulation have since made these assay compliance. The success of these assays revolves around a signal changing in some rapid and measurable away upon

hybridization of a probe to its target(45). By using an excess, the time required for hybridisation of an oligoprobe to its target, especially when the among of that target has been increase by PCR or some other amplifying process, is significantly reduced (45, 49).

In 1991, Holland *et al*. (54)described a technique that was to form the foundation for homogeneous PCR using fluorogenic oligoprobes. Amplicon was detected by monitoring the effect of *Taq* DNA polymerase's 5' → 3'endonuclease activity on specific oligoprobe/target DNA duplexes. The radiolabelled products were examined using thin layer chromatography and the presence or absence of hydrolysis was use as an indicator of duplex formation. These oligoprobes contained 3' phosphate moiety, when blocked their extention by the polymerase, but otherwise had no affect on the amplicon's yield.

An innovative approach used nick-translation PCR in the combination with dual-fluorophore labelled oligoprobes. In the first truly homogeneous assay of its kind, one fluorophore were added to the 5' terminus and one to the middle of a sequence specific oligonucleotide probe. When in such close proximity, the 5' reporter fluorophore (6-carboxy-fluorescein) transferred laser-induced excitation energy by FRET to the 3' quencher fluorophore (6-carboxy-tetramethyl-rhodamine; TEMRA), which reduce the lifetime of the reporter's excited state by talking its excess energy and emitting it as a fluorescent signal of its own. TAMRA emitted the new energy at a wavelength that was monitored but not utilized in the presentation of data. However, when the oligoprobe hybridized to its template, the fluorophores were released due to hydrolysis of the oligoprobe component of the probe/target duplex. Once the labels were separated, the reporter's emissions were no longer quenched and the instrument monitored the resulting fluorescence. These oligoprobes have been called 5'nuclease, hydrolysis or TaqMan oligoprobes.

9. Real-time quantitative PCR

As the reaction progresses, fluorescent signal begins to increase and accumulate with each cycle. A fluorescence threshold (crossing line) can be set above the base line in the exponential portion of the plot. The cycle threshold or C_T value is defined

as the cycle number at which the fluorescent signal from each amplification reaction crosses this fixed threshold. C_T values for a series of reactions containing a known quantity of target can be used to generate a standard curve. Quantification is done by comparing C_T values for unknown sample against this standard curve or against another gene that serve as an internal standard. C_T values are inversely related to the amount of starting template; the higher the starting template in the reaction, the lower the C_T value generated. The C_T value also act as a measure of sensitivity, with lower C_T values indicating greater sensitivity of the reaction.

10. Data evaluation on real-time PCR on LightCycler™

The calculation unit in real-time PCR is a simple specific and characteristic crossing point (CP). For CP determination various fluorescence acquisition methodologies are possible. The "Fit Point Methods" and "Threshold Cycle Methods" measure the CP at the constant fluorescence level (55-58). These constant threshold methods assume that all samples have the same cDNA concentration at the threshold fluorescence. Measuring the level of background fluorescence can be a challenge in real-time PCR reactions with significant background fluorescence variations caused by drift-ups and drift-downs over the course of reaction. Averaging over a drifting background will give an overestimation of variance and thus increase the threshold level (59, 60). The threshold level can be calculated by fitting the interesting line upon the ten-times value of ground fluorescence standard deviation. This acquisition mode can be easily automated and very robust (59). In the "fit point method" the user has discard the uninformative background points, exclude the plateau value by entering the number of log-linear points, and then fits a log-line to the linear portion of the amplification curves. These log lines are extrapolated back to a common threshold line and the intersection of the two lines provides the CP values. The strength of this method is that it is extremely robust. The weakness is that it is not easily automated and so requires a lot of user interaction (57, 60). "Fit Point Method" or "Threshold Cycle Method" can be used on all available platforms with different evaluation of background variability.

The problems of defining a constant background for all samples within one run, sample-to-sample difference in variance and absolute fluorescent value lead to develop a new acquisition modus according to mathematical algorithms. In LightCycler™ software the "Second Derivative Maximum Method is performed where CP is automatically identified and measured at the maximum acceleration of fluorescence (57, 60). The kinetic fluorescence histories of individual curves are different. They show individual background variability (1st phase), exponential and linear growth of fluorescence (2nd and 3rd phase), and finally reaction-specific value (4th phase). The amplification reaction and the kinetic fluorescence history over various cycles is obviously not a smooth and easy function. The mathematical algorithm on which the "Second Derivative Maximum Method" in Roche Molecular Biochemicals software is base is unpublished. But it is possible to fit sigmoidal and polynomial curve models (61-64) with high significant ($p<0.001$) and coefficient of correlation ($r>0.99$), which can be differentiated, and the second-derivate maximum can be estimated (61, 62). This increase in the rate of fluorescence increase, or better called the acceleration of the fluorescence signal, slows down at the beginning of at the 3th linear phase. Therefore the cycle where the second derivative is at its maximum is always between 2nd exponential and 3th linear phase (61).

11. HIV-1 RNA standard

In present day, the HIV-1 RNA standard which used for RNA standard were came from 1) Synthetically HIV-1 RNA standard 2) the cell culture or the supernatant from cell culture which RNA were extracted and quantitated by electron microscope (EM), and 3) biological standard or pool plasma standard which HIV-1 RNA were quantitated by gold standard or confidentially FDA approved HIV-1 RNA quantification assays.

In Thailand, routine laboratories seldom have the known how advance technology such as synthetically RNA standard or viral cell culture to prepare the standard for HIV-1 RNA quantitation. Moreover, the costs of commercial HIV-1 RNA quantitation assays are so expensive.

In 1998, Lin and *et al*.(36) were compared the performance characteristics of enhanced-sensitivity bDNA (ES-bDNA), Roche Amplicor HIV-1 Monotor® tests, NASBA , Baylor College of Medicine (BCM) in-house assay and the National Genetics Institute (culver City, California) or NGI in-house method. The NGI, an established independent laboratory, used HIV-1 RNA quantification' in-house method which the source of the standard were the dilution series prepare from pooled HIV-1 positive sera and plasma. They found that the excellent correlation with nominal concentrations which prepared from BCM. These was the interesting strategy for many low-source countries which was to be used instead of the synthetically HIV-1 RNA standard or the HIV-1 cell culture's standard.