

## CHAPTER 2

### LITERATURE REVIEW

#### 1. The epidemic of HIV-1

According to the United Nations Programme on AIDs (UNAIDS) reported on the global of AIDs epidemic in 2010, approximately 33.3 million people living with HIV worldwide. The epicenter of HIV infection was sub-Saharan Africa with an estimated of 22.5 million infected people. In South and South-East Asia, there were about 4.1 million people living with HIV and 270,000 people were newly infected <sup>(8)</sup>.

In Thailand, the Bureau of epidemiology, Department of Disease Control, Ministry of Public Health reported that there were 372,874 cases of HIV-1 infection and 98,000 cases deaths of AIDS <sup>(1)</sup>.

#### 2. Human immunodeficiency virus type 1

Human immunodeficiency virus was discovered and published by two research teams of the Pasteur Institute, Paris and the Nation Cancer Institute, the Nation Institute of Health (NIH), Maryland. At first, it was isolated from cultured T-lymphocytes derived from a lymph node biopsy of lymphadenopathy patients. Isolated virus was initially named Lymphadenopathy-Associated Virus (LAV) or Human T-cell Lymphotropic Virus Type III (HTLV III). Regarding to the cause of

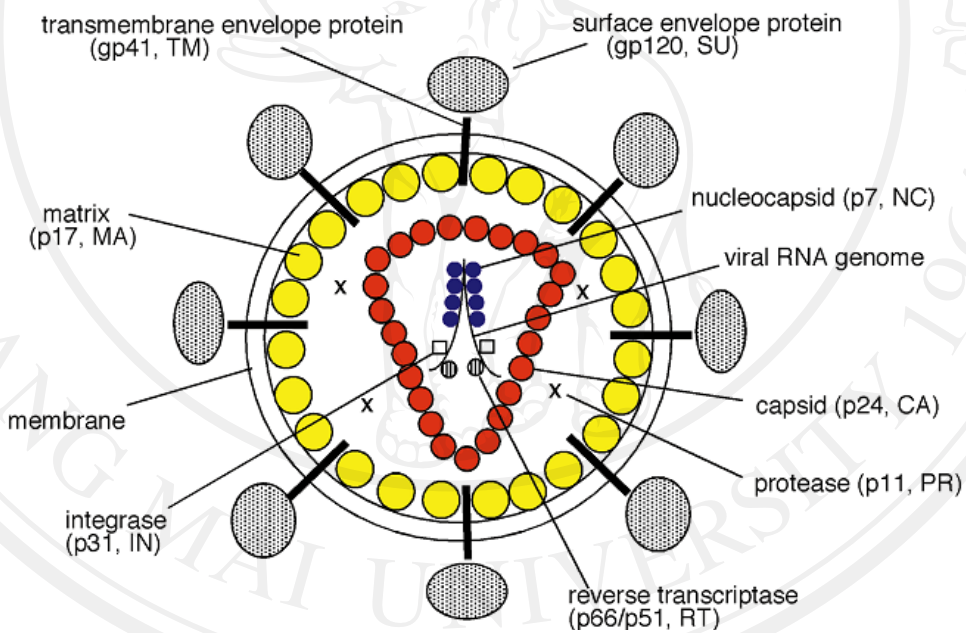
immune defect in infected patients, in 1986, the name was replaced by Human immunodeficiency virus (HIV) <sup>(9)</sup>.

In the present, two types of HIV were defined (HIV-1 and HIV-2). HIV-1 is more virulent and rapid AIDS progression than HIV-2. The AIDS pandemic relates to HIV-1, while HIV-2 is mainly found in West Africa <sup>(9, 10)</sup>.

### 1.1 HIV-1 virion

HIV-1 is classified into family *Retroviridae* and genus *Lentivirus*. The particles are icosahedron structure with an approximately 100-200 nm in size. The viral membrane surrounded by the envelope and knobs. The virus knobs consisted of surface glycoproteins (gp 120) and transmembrane protein (gp 41). Underneath, the surface layer contained matrix proteins (p17) and cone-shaped capsid protein (p24). There were two copies of positive sense single-stranded RNA (+ssRNA) genome that covered by the capsid protein (Figure 1). HIV-1 genome was approximately 9,749 nucleotides in length containing the structural genes such as *gag* and *env*, regulatory genes such as *pol* and long terminal repeat (LTR). These genes were translated into core, envelope and important replication enzymes. Moreover, the accessory genes of virus were elucidated such as Nef, Vpr, Vpu, Vif, Tat and Rev. As the result of virus infected immune cells, the immune system was unable to function efficiently. For this situation, the patients were in at risk of wide range infection such as bacteria, virus, fungi including protozoa and ultimately called acquired immune deficiency syndrome (AIDS) <sup>(9-12)</sup>.

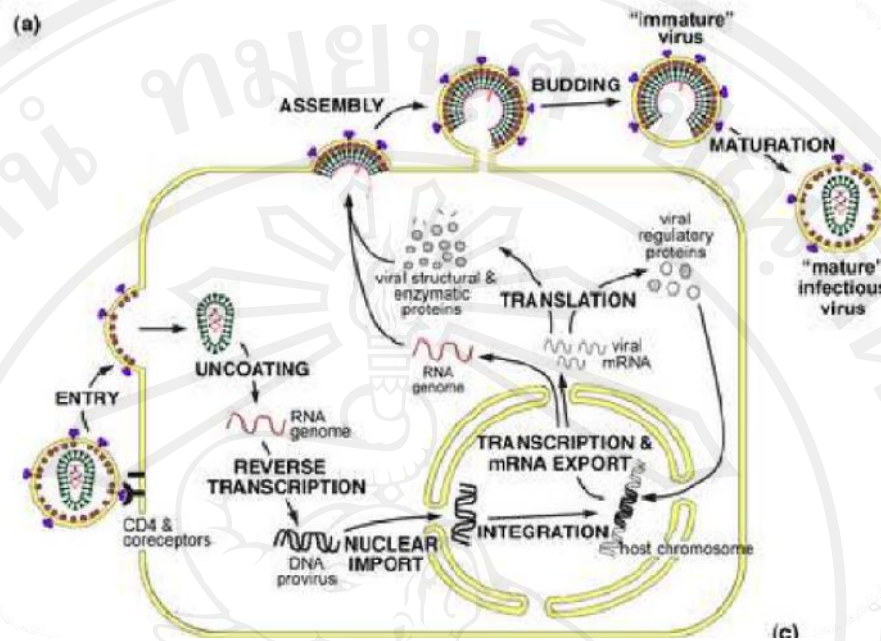
Based on genetic similarities, HIV-1 was divided into three groups including the major group (M), the outlier group (O), and the new group (N). In addition, group M could be divided into 9 clades or subtypes (A, B, C, D, F, G, H, J and K) including the circulating recombinant forms (CRFs). Most HIV-1 found in Thailand is subtype B and CRF01\_AE.



**Figure 1** The schematic of Human immunodeficiency virus type 1 (HIV-1) structure <sup>(13)</sup>.

## 1.2 HIV-1 replication<sup>(9, 10)</sup>

The major cell receptor for viral attachment is CD4 receptor that usually found in helper T-lymphocytes and macrophages. HIV-1 infects into these cells through the specific recognition of viral gp120 enveloped protein and CD4 domain. In addition, CXCR4 and CCR5 co-receptors are necessary for stably viral attachment on cell surface of T-lymphocytes and macrophages, respectively. After binding, gp120 and gp 41 have conformation change and cell membrane fuses together, resulting in virus entry and uncoating. Viral RNA genome is converted by using self-reverse transcriptase enzyme and the complementary strand is synthesized by using host cell machinery. Double-stranded DNA (dsDNA) containing long terminal repeats (LTR) at the end of genome is transported to nucleus of the cell and integrated into the host genome by using viral integrase (p32). Transcription of viral RNA is utilized from host cell enzyme (cellular RNA polymerase II). Viral messenger RNAs (mRNAs) are transported to the cytoplasm and translated into proteolytic enzymes and structural proteins using host cell ribosomes. To form mature envelope proteins at the surface of infected cell, enveloped protein is translate and cleaved into gp 120 and gp 41. These two proteins are formed together by non-covalent bonding. Assembled HIV-1 virus are located at cellular membrane and released from infected host cell by using budding system. To form mature particle, gag and gag-pol proteins are finally cleaved by protease. HIV-1 replication cycle is illustrated in figure 2.



**Figure 2** The life cycle of HIV-1 in host cell <sup>(14)</sup>.

### 1.3 Progression of HIV-1 infection <sup>(9, 10)</sup>

Three phases of virus infection are indicated; primary infection, chronic asymptomatic infection and AIDS stage.

#### 1.3.1 Primary infection

During the early stage of HIV-1 infection, an increasing of viral load was observed in the blood, central nervous system and lymphatic system. Asymptomatic infection might be seen in some infected individual. The most common symptoms comprised of an illness resembling fever or influenza, which generally referred to as

acute retroviral syndrome (ARS). This period was usually occurred in two to six weeks after infection.

### **1.3.2 The chronic asymptomatic phase of infection**

After virus infected into the host cells, it was recognized and cleared by the immune response. In this period, signs and symptoms might be disappeared and could be defined as typical progressors. The average life span of non-treated patients was approximately 8-10 years in which dependent on characteristics of host immunity and nature of virus. Rapid progressors was defined in infected individuals whom AIDS could be developed within five years and about 10-20% was found in this group. In contrast, 5-15% of infected people could survive with AIDS for over 15 years was called slow progressors. Only 1% developed no sign of AIDS was indicated as long term nonprogressors.

### **1.3.3 AIDS stage**

Without any intervention, AIDS is the last phase of the disease progression. The patient would die within two to three years. The most common characteristic of AIDS could be defined by the infection with multiple pathogenic micro-organisms since a huge rising of viraemia and impaired immune response. When the CD4+ cell counts was analyzed and found to be less than 50 cells/ $\mu$ l, a number of opportunistic infections were increased and infected patients are at risk of death. The most common infectious diseases associated with AIDS were penicilliosis, toxoplasmosis, tuberculosis and pneumonia. In addition to microbial infections, the patients were

also suffered from malignancies such as systematic non-Hodgkin's lymphoma. Moreover, wasting syndrome, a loss of body weight more than 10% associate with fever or diarrhea, usually occur in many AIDS patients. For longer survivorship and decreased mortality of severe immune-compromised patients, the management with antimicrobial prophylactic regimens and antiretroviral therapy were applied.

#### **1.4 HIV-1 transmission and prevention** <sup>(10, 11)</sup>

Three major routes were important for HIV-1 virus transmission, sexual activity, transmission through infected blood and vertical transmission. Infected CD4+ lymphocytes and free viral particles from HIV-1 infected patient could be transmitted to sexual partner during sexual activity. In addition, the data revealed that HIV-1 was equally spread in both heterosexual and male homosexual activity. Hypodermic needles contaminated with HIV-1 virus were caused of blood transmission. Risk of virus infection was also observed among the blood transfusion, accidental needle stick injury in clinical practitioners and needle sharing in drug addict. The mode of HIV-1 transmission though infected mother to child during pregnancy was studied and approximately 15-45% of child was transmitted in the absence of any interventions. However, this rate can be reduced to levels below 5% with effective interventions. Breast feeding was defined to involve in an increasing risk of virus transmission from infected mother to their child.

A variety tools and procedures were prescribed for prevention of HIV-1 infection. Infected individual could prevent sexual transmission by avoiding of exchanged body fluid with infected people. Health education and safer sex advising

have been declared for reduction of HIV transmission worldwide. Screening of HIV-1 infection in blood donors, blood products and disposable materials used were provided for recipient's prevention through infected blood. Anti-retroviral drug treatment must be promptly administered in post-exposure prophylaxis of accidental person. Nowadays, the appropriated intervention of anti-retroviral drug was established and taken part to reduce the risk of mother to child transmission of HIV-1.

### **3. Laboratory diagnostic for HIV-1 infection**

During infection, several markers can be used for HIV virus detection. Viral RNA was able to detect in plasma within 2 weeks or 10-12 days. Viral RNA was increasing up to about 1 million copies of RNA/ml within a couple of months. Meanwhile, the immune responses are developed and able to control viral replication resulting in the reduction of viral RNA<sup>(15)</sup>. As early as 11-13 days after infection, viral p24 antigen can be detected later. The antigen concentration highly remained and could be able to measure for about one month and a half after infection. In the window period that was characterized as the absence of HIV-1 antibodies, the viral RNA or p24 antigen and variation of CD4+ T lymphocyte level might be measurable<sup>(15, 16)</sup>. HIV-1 specific Ig M antibodies were first appeared within first three weeks after infection and specific Ig G antibodies were subsequently appeared about 3-4 weeks depending on individuals' immune response. Ig G antibodies could reduce in around 10-12 weeks<sup>(15, 17-19)</sup>. Several tools for diagnosis were based on both detection of HIV specific antibodies, viral antigen and viral nucleic acid.



### 3.1 Antibody tests

The most common method for diagnostic of HIV infection in adult and over 18 months old children were HIV antibodies. After infection, antibodies could be detected within three to six weeks. In general, the seroconversion of most infected people was found within 12 weeks. Individuals who initially examined and indicated negative result should be followed up in 3 months for confirming of viral infection<sup>(20)</sup>. Presently, highly sensitive serological testing is required for screening such as an enzyme-linked immunosorbent assay or ELISA and sample should be confirmed the presence of HIV-1 antibodies with highly specific test (usually Western blot analysis)<sup>(15, 21, 22)</sup>.

### 3.2 Virus culture

Peripheral blood mononuclear cells or PBMCs was utilized for HIV-1 virus culture. After mixing of patient infected cells with uninfected PBMCs, the culture further stimulated with mitogens such as phytohemagglutinin (PHA) and interleukin-2 (IL-2). Within one or two weeks, the culture supernatant is collected for determination of p24 antigen or reverse transcriptase (RT) activity. However, it was defined to be negative when the cell culture was incubated up to 4 weeks. In addition, the quantitation of HIV infection in plasma was performed by culturing serial dilutions of the patient's plasma with fixed number of PHA-stimulated uninfected PBMCs. The infectious titer of HIV in plasma was then calculated. Although virus culture technique was reliable and always used as reference method, cost and labor-intensive might be considered<sup>(21, 23, 24)</sup>.

### 3.3 HIV-1 antigen assays

HIV-1 p24 antigen was used as target for determination of virus in serum. Several commercial kits based on the enzyme immunoassay (EIA) was applied to detect the presence of free and bound form of p24 protein. Either monoclonal or polyclonal antibodies were utilized as capture antibody. Nowadays, HIV p24 antigen testing is one of the test used for early stage detection of virus infection in neonatal diagnosis <sup>(21)</sup>.

### 3.4 Viral nucleic acid detection

Molecular biology techniques were applied for determination of type, genetic subtypes and early detection of HIV. Based on nucleic acid amplification, polymerase chain reaction (PCR) is widely used for viral detection in both pro-viral DNA in leukocytes and viral RNA in cell-free compartment <sup>(20)</sup>. During acute infection, viral RNA level is increased and could be detected within two weeks before seroconversion. Currently, several PCR detection methods were established including branched DNA (bDNA), nucleic acid sequence-based amplification (NASBA), ligase chain reaction (LCR) and real-time PCR. Recently, the Aptima HIV-1 RNA qualitative assay (Gen-Probe) is approved by FDA for diagnostic of acute or primary HIV infection. The PCR technique preferred many advantages in rapid, highly sensitive and specific for HIV-1 detection whereas personal skill, stringent quality control and special laboratory equipment are required <sup>(15, 20, 21, 25)</sup>.

#### **4. The monitoring test for HIV-1 infection**

Many tests were developed for monitoring of disease progression in infected individual. Currently, CD4+ lymphocyte count and HIV-1 viral load in plasma were applied<sup>(4)</sup>.

##### **4.1 CD4+ T-lymphocyte counts**

As a target of HIV-1 virus infection, the level of CD4+ T-lymphocytes is a useful marker for monitoring the immune system during the course of infection. The cell counts could be utilized for prediction of disease progression, determined the stage of disease and required for opportunistic infection prophylaxis. In addition, CD4+ cell counts are used to determine the timing, initiation of antiretroviral therapy and responsibility of the therapy<sup>(3, 21)</sup>. Flow cytometry is widely used for counting of CD4+ lymphocytes<sup>(26)</sup>. For the quantification, both automated methods and low cost manual method are performed. There is some variability from difference laboratory. Thus, the clinician should select the same protocol for patients monitoring. The Center for Disease Control (CDC) indicated that the definition for AIDS is infected individual with absolute CD4+ lymphocyte counts less than 200 cells per microliter, even symptomatic or not<sup>(21)</sup>.

##### **4.2 Viral load testing**

Viral load is defined as quantitation of HIV-1 RNA in plasma and used as a standard tool for viral replication within the infected individual indication, monitoring of the outcome of antiretroviral therapy and disease progression<sup>(3, 4, 26, 27)</sup>.

Approximately 11 days post infection, viral RNA in plasma could be detected. Prior to develop the immune response, the rising of HIV viral load was observed during the primary infection. Within 6-9 months, the level of HIV RNA was stable since the host immune response and level of viral replication was balance <sup>(21, 22, 28)</sup>. A successful anti-retroviral therapy was defined while a 100-fold (2 log<sub>10</sub>) or greater of HIV RNA plasma level was decreased. In contrast, the unchangeable level of RNA virus was observed within 2-4 months after anti-retroviral treatment would be defined as non-responder or resistance might be occurred <sup>(22)</sup>.

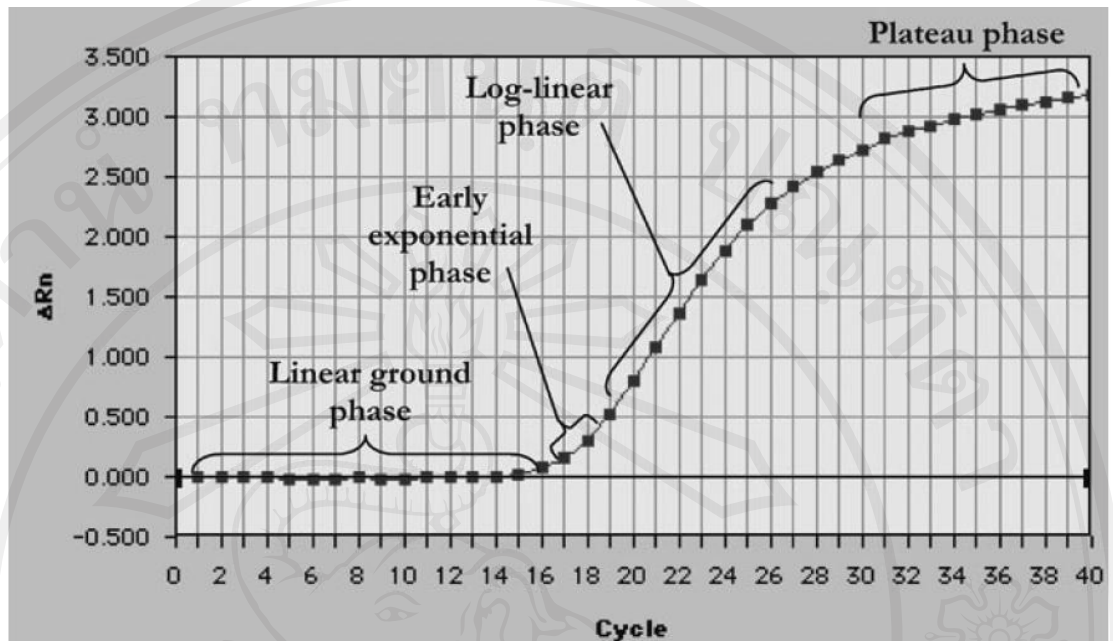
Furthermore, the level of HIV RNA was enabled for risk assessment of mother to child transmission during pregnancy or parturition <sup>(21, 29)</sup>. Several methods for viral load assays was commercially available including the reverse transcription-polymerase chain reaction (RT-PCR; Amplicor™ HIV-1 Monitor Test, Roche DiagnosticSystems, Pleasanton, CA), the nucleic acid sequence-based amplification (NASBA:NucliSens™ HIV-1 QT Test, OrganonTeknika, Bostel, The Netherlands), the signal amplification methodology or branched DNA (bDNA) technique (Quantiplex™ HIV-1 RNA test, Bayer Diagnostics, Emeryville, CA) <sup>(27)</sup>. These commercial assays have widely used as gold standard methods for quantitative of HIV-1 RNA in plasma.

The commercial HIV viral load assays have been developed and currently used in United States and Thailand such as Amplicor HIV-1 Monitor test (Roche), Versant/Quantiplex HIV-RNA or bDNA (Chiron/Siemens), NucliSens™ HIV-1 QT Test, Abbott Real-time HIV-1 and COBAS AmpliPrep/COBAS TaqMan HIV-1 test <sup>(20)</sup>.

## 5. Real-time polymerase chain reaction

Real-time polymerase chain reaction (real-time PCR) is a modified technique from conventional PCR which was first introduced by Higuchi and his colleague<sup>(30, 31)</sup>. It was the simultaneous combination of sequence amplification and detection. This technique required high throughput system for data collection in each cycle of the process. An increasing of target gene amplification was correlated with the fluorescence intensity was detected<sup>(30-32)</sup>. Thus, real-time PCR products are proportional to the number of template before the starting of PCR process<sup>(33, 34)</sup>.

The real-time PCR reaction can be divided into four phases including initial ground phase, early exponential phase, linear-log phase, and plateau phase. At the beginning of reaction, the background fluorescence emission was observed and defined as ground phase. This phase was calculated as baseline fluorescence. The early exponential phase was found while the fluorescence signal reached over the cut-off or threshold level. The cycle number that the fluorescence intensity was higher than threshold was designated as cycle threshold ( $C_t$ ). Optimal PCR amplification with PCR product duplication is occurred in log-linear phase. Finally, plateau phase was observed when the PCR components were limit<sup>(32, 35, 36)</sup>. The phases of PCR amplification curve was shown in figure 3.



**Figure 3 Phases of PCR amplification curve** <sup>(32)</sup>.

Many advantages of real-time PCR were revealed including highly sensitive, specific and omitting of post-PCR process. Additionally, real-time PCR could be used for quantitative analysis. It allowed precise detection of specific nucleic acids even in very low concentration of starting materials. However, sophisticated and high cost equipment was the major disadvantage for real-time PCR <sup>(30, 32)</sup>.

## **5.1 Fluorescent chemistries for monitoring real-time amplification**

Several fluorescent chemistries were used for real-time PCR detection.

### **5.1.1 DNA binding dyes**

According to the intercalating property into double-stranded DNA, several fluorescent dyes had been used in real-time PCR technique. SYBR Green I was one of the fluorescent dyes that could bind into the minor groove of DNA and fluoresced when the accumulation of amplified fragment was increased<sup>(34)</sup>. The greater the amount of double stranded-DNA concentration presents in reaction, the greater the amount of fluorescent intensity. It was found little background fluorescence in normal situation but emitted over 1,000 fold greater fluorescence signal while double stranded DNA was increased during polymerization<sup>(33, 34, 37)</sup>. Mis-priming of primers used might cause of false positive result and should be concerned<sup>(33, 38)</sup>.

### **5.1.2 Hybridization probes**

The hybridization probes technique used for real-time PCR was dependent on the two sequence-specific probes in which one of these was labeled with fluorescent acceptor dye at the 3' end and the other was labeled with fluorescent donor dye at the 5' end of target fragment. During denaturation step, both probes were separated and fluorescent donor dye was unable to emit. In the annealing step, both probes hybridized to specific sequence of DNA next to each other in a head-to-tail arrangement. The donor dyes was induced and excited the fluorescence resonance

energy transfer (FRET) to the acceptor dye. The acceptor dye was subsequently induced and emitted the fluorescence intensity. This method allowed detecting the signal only in a result of hybridization of two independent probes to specific target sequence. The fluorescent intensity was proportional to the amount of amplified products<sup>(32, 33, 35)</sup>.

### 5.1.3 Hydrolysis probes

The principle of hydrolysis probe was utilized from the 5'-3' exonuclease activity on sequence specific probe labeled with two fluorescent dyes consisting of reporter dye on the 5' end and a quencher dye on the 3' end. This probe was most commonly referred to TaqMan probes. In the normal stage, the fluorescence intensity of reporter dyes was suppressed by the quencher dye when the probe was in proximity intact. To generate a signal, double stranded

DNA were separated and complementary strand of DNA was simultaneously annealed with the primers and probe at downstream of primer site. During extension step, the 5'-3' exonuclease activity of Taq polymerase was utilized to cleave TaqMan probe. The dissociation of the fluorescent reporter dye from quencher dye was resulting in an increasing of fluorescence reporter dye emission and this signal was proportionated to the amount of PCR product<sup>(7, 32, 34, 35)</sup>. Various fluorescent reporter dyes were currently used including 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), hexacholo-ro-6-carboxy-fluorescein (HEX), or VIC. The most common quencher dyes was 6-carboxytetra-methyl- rhodamine (TAMRA) or 4-(dimethylaminoazo) benaene-4-carboxylic acid (DABCYL)<sup>(33)</sup>.



#### 5.1.4 Molecular beacon

Real-time PCR using specialized molecular beacon probe was published. This probe was designed as hairpin or stem-and-loop structure that covalently bound fluorescent reporter dye and quencher dye at the end of either single-stranded DNA molecule. In the normal stage, the fluorescent dye and quencher dye were attached to each other resulting in a quenching of fluorescent emission. When the loop portion of the probe complementary bound to target sequence during annealing step, the quencher and reporter dye were far separated and an emitting of reporter dye fluorescent intensity indicated the amount of amplification product in each cycle. This molecular beacons probe was in an intact form and could be rehybridized in each annealing step of amplification reaction <sup>(7, 32, 33, 35)</sup>.

### 6. Quantitative real-time PCR

The method that commonly used for quantification of amplified product using real-time PCR was standard-curve or absolute quantitation <sup>(33)</sup>.

Standard-curve was generally constructed by using a linear relationship between cycle threshold (Ct) and a set of known standards copy number in serial dilution. The concentration of unknown target was determined by measuring the Ct and compared to the amount of known standard in a standard curve. Plasmid constructed and synthetic single-stranded oligonucleotides were usually used as a source of known sample. The standard DNA or RNA should have the same primer binding sites as found in the target sequence of interest and the same product size as target was possibly produced <sup>(30, 32, 33)</sup>.

To monitor HIV-1 viral load in resource-limited settings, an inexpensive in-house real time PCR assays has been developed. Real-time PCR assay using many kinds of technologies for detection such as TaqMan<sup>(39, 40)</sup>, SYBR green I dye<sup>(41)</sup> and double-stranded primer<sup>(42)</sup> were applied. An internal control was developed and utilized in several of experiments<sup>(42-45)</sup>.

#### **7. PCR inhibitors<sup>(46-48)</sup>**

A variety of substances in clinical samples were published to be nucleic acid amplification inhibitors. Bile salts and complex polysaccharides in feces, heme in blood and urea in urine were revealed to inhibit the PCR reaction. Furthermore, hemoglobin and lactoferrin in erythrocytes and leukocytes were reported as PCR-inhibitors. In a recent study, the mechanism of PCR inhibition was studied and indicated that a variety of inhibition mechanisms occurred during PCR process. In some inhibition mechanisms, it was found that amplicon size and melting temperature were related.