CHAPTER 3

STUDY DESIGN, MATERIALS AND METHODS

1. Study design

The flow chart of study design used in the study was shown in figure 4.

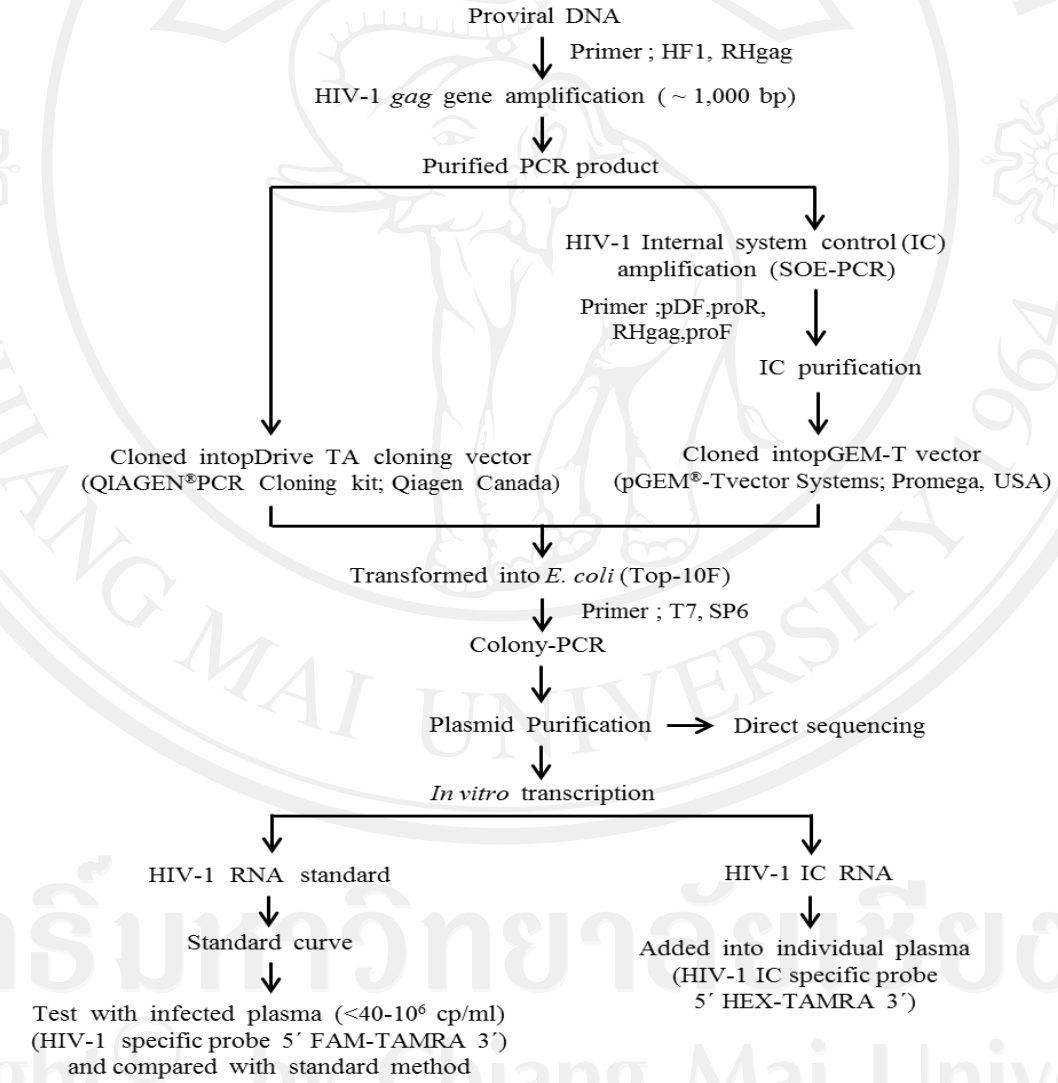

Figure 4 The study design used in this study.

2. Materials

2.1 Plasma samples

A total of 232 plasma samples consisting of 40 samples of HIV-1 sero-negative plasmas derived from Blood Banking Unit, Red Cross Society, Chiang Mai and 192 HIV-1 positive samples attending at Clinical Microbiology Service Unit, Faculty of Associated Medical sciences, Chiang Mai University during February 2011 to September 2011. HIV-1 negative plasma samples were tested and identified as HIV-1 serological negative according to WHO criteria. Of these positive samples, the viral load has been previously determined by CobasAmpliprep/CobasTaqman HIV-1 test (Roche diagnostics GmbH, Germany) in which comprising of 19 samples of the viral RNA load less than 40 copies/ml, 31 samples of 40-10^2 copies/ml, 40 samples of 10^2-10^3 copies/ml, 31 samples of 10^3-10^4 copies/ml, 30 samples of 10^4-10^5 copies/ml and 41 samples of 10^5-1.7x10^6 copies/ml. All left over plasmas were stored at -70^{o}C until analysis.

2.2 Primers and Probes

The primers and probes were designed according to the conserved *gag* region of HIV-1 subtype AE and B by Associated Professor Dr. Wasun Chantratita at Ramathibodi Hospital, Faculty of Medicine, Mahidol University. TaqMan probe specific for HIV-1 genome was commercially labeled with 6-carboxy fluorescein FAM (6-FAM) as a reporter dye at the 5′ end and a quencher dye with 6-carboxy

tetramethylrhodamine (TAMRA) at the 3′ end. Therefore, the internal system control probe was modified from TaqMan HIV-1 specific probe with the same type and number of nucleotides whereas different in the position (scrambled). The internal control probe was labeled with Hexa-chloro-6-carboxy fluorescenin (HEX) at the 5′ end and TAMRA at 3′ end. HIV-1 probe were initially blasted for correctly checking in GenBank database before commercially synthesized. All primers and probes sequences used in this study were shown in Table 1. The position of primers and probes were illustrated in figure 5.

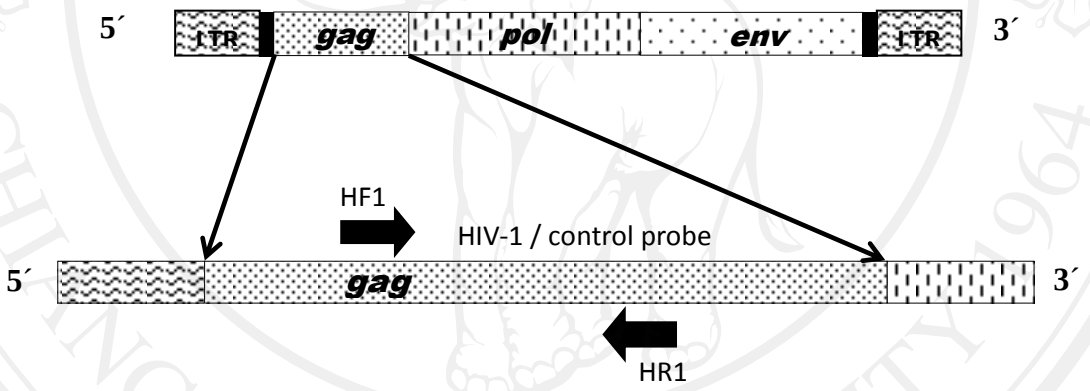

Figure 5 The primers and probes binding site on HIV-1 genome used in this study.

2.3 Plasmid vectors

2.3.1 pGEM®-T Vector Systems (Promega; USA) (Figure 6)

2.3.2 pDrive Cloning Vector (QIAGEN® PCR Cloning kit; Qiagen, Canada) (Figure 7)

Table 1 The nucleotide sequence of primers and probes used in this study.

Primer and Probes	Position	Sequence	Method
RH-*gag*	2264-2281	5' GCCAAAGAGTGATTTGAG 3'	3.3,3.5
HF1	1311-1332	5' TACCCATGTTCTCAGCATTATC 3'	3.3,3.11
HR1	1398-1420	5' GATGGTTTCTTTTAACATTTGCA 3'	3.11
pDF		5' ACTCACTATAGGGAAAGC 3'	3.5
Pro-F		5'CTACAATAGACTACACTAGCATAGCCTAA ATATAGTGGG 3'	3.5
Pro-R		5'TAGTGTAGTCTATTGTAGCTACCCTCTGATAATG CTGA 3'	3.5
T7		5' CTAATACGACTCACTATAG 3'	3.7
SP6		5' CATTTAGGTGACACTATAG 3'	3.7
HIV-probe	1339-1366	5'FAM- AGCCACCCCACAAGATTTAAATAT GATG-TAMRA 3'	3.11
Internal system control-probe	1339-1366	5'HEX-TAGCTACAATAGACTACACTAGCA TAGC-TAMRA 3'	3.11

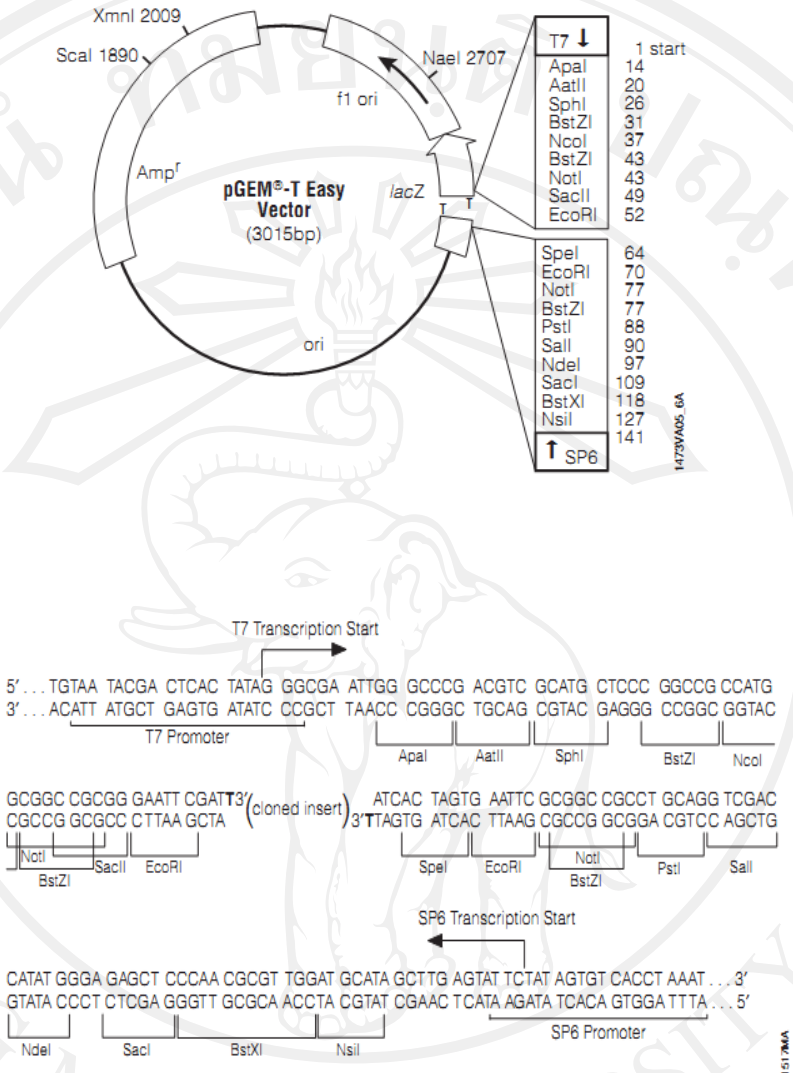

Figure 6 Map and multiple cloning site of pGEM®-T Vector (Promega; USA)[49].

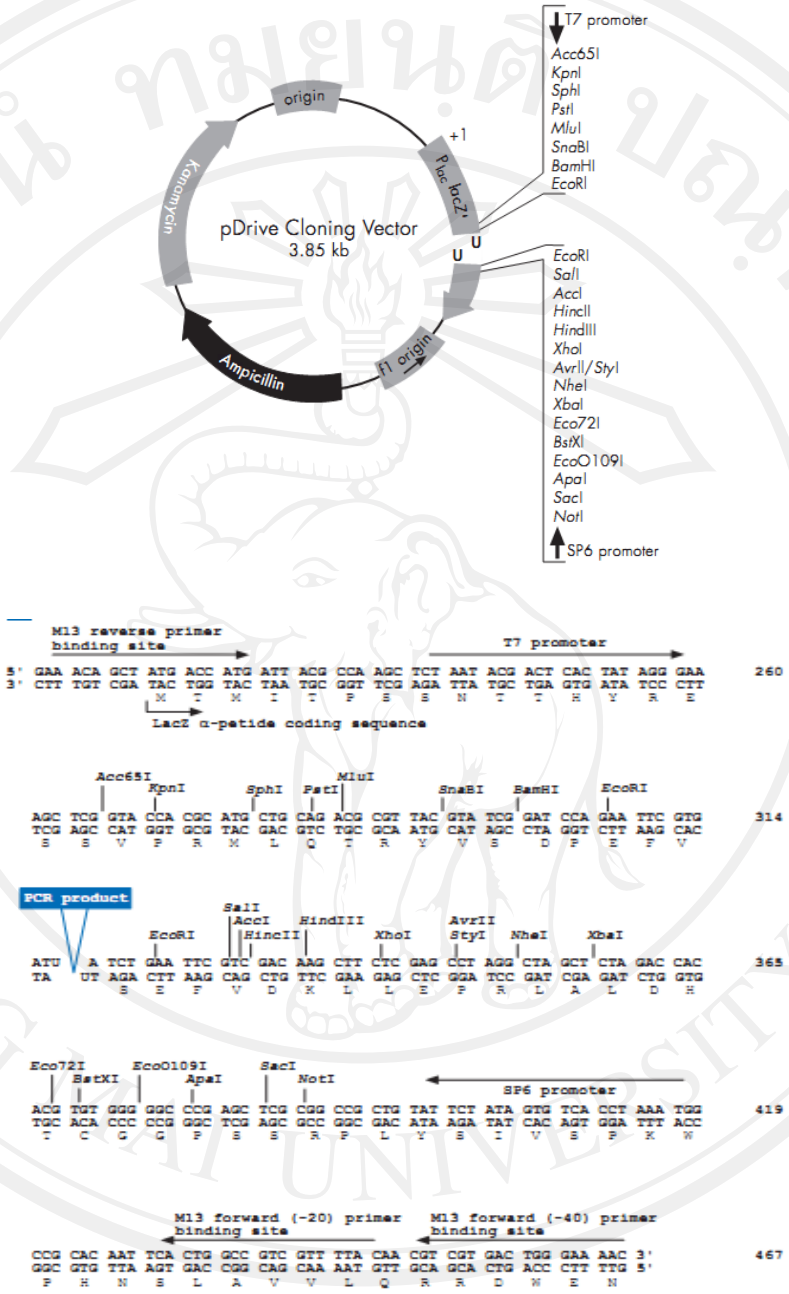

Figure 7 Map and multiple cloning site of pDrive Cloning Vector (QIAGEN® PCR Cloning kit; Qiagen Canada) [50].

2.4 Commercial kits

Several commercial kits were used in this study. All reagents were molecular biology grade (Table 2).

Table 2 Lists of commercial kit used in this study.

Commercial kit	Supplier
High Pure Viral RNA kit	Roche DiagnosticGmbH, USA
NucleoSpin®Extract II	MACHEREY-NAGEL GmbH &Co., Germany
NucleoSpin®Plasmid	MACHEREY-NAGEL GmbH &Co., Germany
RiboMAX™ Large Scale RNA Production Systems-SP6 and T7	Promega, USA
RNA-direct™ Real time PCR Master Mix	TOYOBO, Japan
i-Taq™DNA Polymerase kit	iNtRON Biotechnology, Korea

3. Methods

3.1 Viral RNA extraction

HIV-1 RNA was extracted from plasma using high pure viral RNA kit as followed by the manufacturer's instruction. Briefly, 200 µl of plasma was added into 400 µl of binding buffer supplemented with poly (A), mixed and transferred into filter tube. After centrifugation at 8,000 g for 15 sec, the flow through was discarded and 500 µl of inhibitor removal buffer was added. After washing twice with wash buffer, viral RNA was eluted and stored at -80 ^{o}C until analysis. All solutions were supplied from the kit.

3.2 *E. coli* competent cell preparation

The *E. coli* competent cells (Top-10F strain) used in this study was kindly provided from Assist. Prof. Dr. Ratchada Cressey, Division of Clinical Chemistry, Department of Medical Technology, Faculty of Associated Medical Sciences. The competent cell preparation was performed according to established procedure [51]. Firstly, *E. coli* strain Top-10F was overnight cultured in 2 ml of LB broth at 37 ^{o}C, 250 rpm. Bacterial cell suspension was continuously cultured in 100 ml of LB broth (1:100) at 37 ^{o}C, 250 rpm until the absorbance at 600 nm was reached to 0.25-0.3. The bacterial culture was placed on ice for 15 minutes and centrifuged at 3,300 g, 4 ^{o}C for 10 minutes. Cell pellet was subsequently suspended with 30 ml of 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. Finally, cell pellet was resuspended with 0.1 M $CaCl_2$ and 15% glycerol, aliquoted and stored at -70 ^{o}C until used.

3.3 The HIV-1 *gag* gene amplification from pro-viral DNA

HIV-1 proviral DNA was extracted from HIV-1 positive dried whole blood and used as template for HIV-1 *gag* gene amplification by PCR using HF1 and RH-*gag* primers (Table 1). PCR product was initially calculated to be 956 bp. The PCR reaction was performed in 25 µl reaction mixture containing 10x PCR buffer with $MgCl_2$, 75 mM dNTP, 10 pmol of each primer and 2.5 units of i-Taq^{TM} DNA polymerase. The reaction profiles were as followed: pre-denaturation at 94 ^{o}C for 30 sec, 40 cycles of 94 ^{o}C for 30 sec, 50 ^{o}C for 30 sec, 72 ^{o}C for 1 min and final extension at 72 ^{o}C for 5 min. The amplified product (10 µl) was electrophoresed in

1.5% agarose gel, stained with ethidium bromide and analyzed by using UV transilluminator.

3.4 DNA purification from agarose gel

The amplified product of HIV-1 *gag* gene (experiment 3.3) was purified from agarose gel using NucleoSpin®Extract II kit as followed by manufacture's instruction. Briefly, the amplified fragment was carefully excised from agarose gel and weighed. A 200 µl of buffer NT was added for each 100 mg of gel slice and incubated at 50 ^{o}C until it completely dissolved (5-10 minute). After that the aqueous solution was applied into the NucleoSpin® Extract II column and centrifuged at 11,000 g for 1 min. The membrane was washed using 600 µl of Buffer NT 3 and centrifuged at 11,000 g for 1 min. To remove trace of ethanol in washing buffer, the membrane was repeatedly centrifuged at 11,000 g for 2 min. Finally, bound DNA was eluted using 20 µl DNase and RNase free water and kept at -70 ^{o}C until used. The amount of DNA was quantified by UV spectrometer at wavelength 260 nm (1 OD equal to 50 µg/ml DNA).

3.5 Internal system control construction using splicing-overlapped extension PCR

Splice overlapped extension PCR (SOE-PCR) technique was used for internal system control construction in this study. Complementary overlapped primers, ProF and ProR, were designed by modification of HIV-1 specific probe with scrambled sequence at 5′ and 3′ overhang, respectively. Two overlapped fragments were

separately amplified and combined together using forward outer primer (HF1) and reverse outer primer (RH-*gag*) as shown in figure 8. HIV-1 proviral DNA was used as template for amplification by using the same PCR mixture and profile as the method 3.3. The combined PCR product (1,043 bp) was electrophoresed in 1.5% agarose gel, stained with ethidium bromide and analyzed by using UV transilluminator. The DNA quantitation was determined using UV spectrophotometer at wavelength 260 nm.

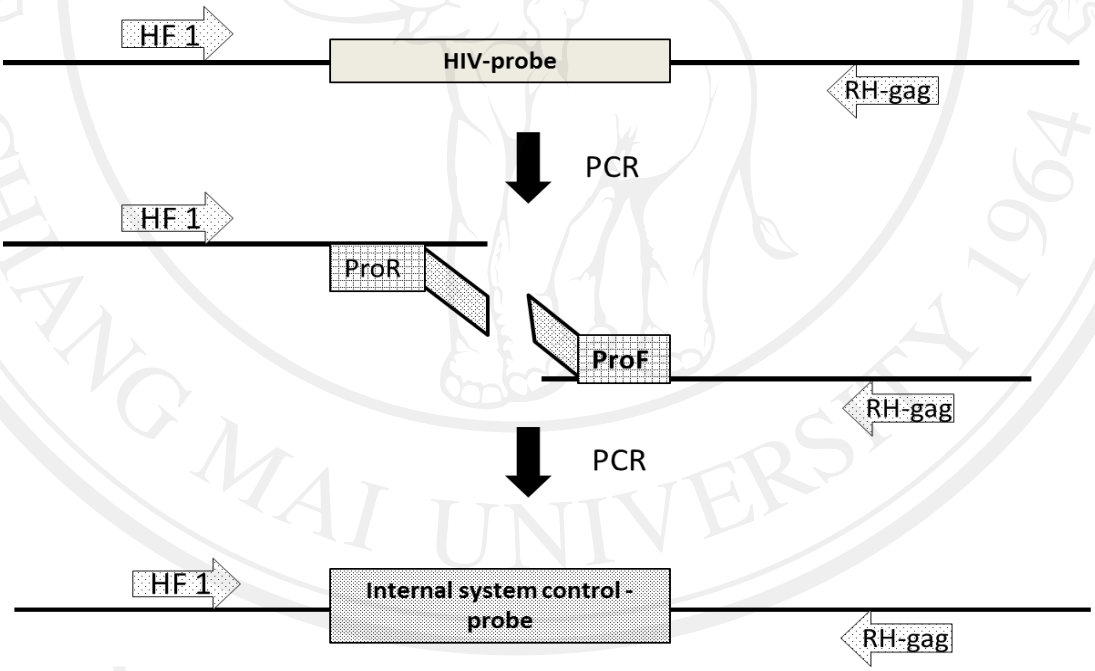

Figure 8 Illustration of internal system control construction by splice-overlapped extension PCR (SOE-PCR).

3.6 The PCR cloning of standard HIV-1 *gag* gene and internal system control

3.6.1 DNA ligation

The amplified product of HIV-1 *gag* gene and internal system control were gel purified and ligated into the TA cloning vector, pDrive and pGEM®-T, respectively. Shortly, approximately 100 ng of purified fragments were mixed with ligation mixture containing 50 ng/µl of either pDrive or pGEM®-T vector and 2x ligation master mix (supplied from kit). The reaction mixture was incubated at 16 ^{o}C for overnight.

3.6.2 DNA transformation

One to ten microlitre of both ligation products were transformed into *E. coli* top-10F competent cells. The heat-shock transformation procedure was conducted as previously described [51]. First of all, the ligation product was mixed with *E. coli* Top-10F competent cell and incubated on ice for 30 min. The mixture was immediately heated at 42 ^{o}C for 50 sec and further incubated on ice for 2 min. Five hundred microliter of SOC medium was added and the mixture was incubated at 37 ^{o}C, 250 rpm for 3 hours. Cell suspension was gently centrifuged at 12,000 rpm for 1 min. After discarded the supernatant, the cell pellet was resuspended with 100 µl LB medium, plated on LB agar containing either kanamycin (30 µg/ml) or ampicillin (100 µg/ml) and incubated at 37 ^{o}C for 16-20 hours.

3.7 Colony PCR for selection of transformant

The positive transformed *E. coli* that carried the target cloning vector was selected by colony PCR using T7 and SP6 primers (Table 1). Sterilized tooth pastes were used to pick up the bacterial colonies and mixed with freshly prepared PCR mixture as followed by method 3.3. PCR profile was modified by pre-denaturation at 94 ^{o}C for 30 sec and 40 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 50 ^{o}C for 30 sec, extension at 72 ^{o}C for 70 sec and final extension at 72 ^{o}C for 5 min. The amplified products was electrophoresed in 1.5% agarose gel, stained with ethidium bromide and analyzed by using UV transilluminator. The PCR products of inserted HIV-1 *gag* gene in pDrive cloning vector and internal system control in pGEM-T vector were 1,152 and 1,213 bp, respectively.

3.8 Plasmid purification and sequencing

The plasmid DNA that carried target insert gene was purified using NucleoSpin® Plasmid kit. An isolated colony of *E. coli* positive clone was subcultured in 3 ml LB broth containing either kanamycin (30 µg/ml) or ampicillin (100 µg/ml) and incubated at 37 ^{o}C for overnight. After centrifugation, cell pellet was resuspended in 250 µl of buffer A1 and 250 µl of buffer A2. The lysate was mixed and incubated at room temperature for 5 min (the cleared lysate could be observed in this step while the bacterial cells were completely lysed). Three hundred microliter of buffer A3 was added and the mixture was centrifuged at 11,000 g for 5 min. Approximately 650 µl of supernatant was carefully loaded into the set column and centrifuged at 11,000 g for 1 min. The plasmid DNA was washed with 600 µl of

buffer A4 and centrifuged at 11,000 g for 1 min. The column was repeatedly centrifuged to remove trace of ethanol at 14,000 g for 2 min and eluted with 50 µl nuclease free water. DNA sequencing was subsequently performed for integrity determination of insert gene.

3.9 *In vitro* transcription for HIV-1 *gag* RNA and internal system control RNA production

HIV-1 *gag* RNA was *in vitro* transcribed and applied for external standard curve construction. In addition, internal system control RNA was individually provided and utilized for controlling of the false undetectable results caused by PCR inhibitors in plasma.

3.9.1 Linearized plasmid DNA

Prior to transcription, approximately one microgram of plasmid DNA were linearized by using 20 units of *Sal* I restriction enzyme. The reaction was incubated at 37 ^{o}C for overnight.

3.9.2 DNA precipitation

Linearized plasmid DNA was precipitated according to the established procedure [51] by gently mixing with one-tenth volume of 3 M sodium acetate, pH 5.2 and approximately two times of absolute ethanol. The solution was incubated at -70 ^{o}C for 1 hour and centrifuged at 12,000 rpm for 10 min. Supernatant was discarded. The DNA pellet was washed with 500 µl of 70% ethanol and centrifuged

at 12,000 rpm for 10 min. Supernatant was completely discarded and allowed to dry at room temperature for 5-10 min. Finally, the pellet was resuspended with 20 µl of nuclease-free water and available for in vitro transcription.

3.9.3 RNA synthesis by *in vitro* transcription

In this study, HIV-1 *gag* RNA was in vitro transcribed using a commercial kit, RiboMAX™ Large Scale RNA Production Systems-SP6 and T7. Reaction was performed according to manufacturer's instruction. Briefly, five to ten microgram of linearized plasmid DNA was mixed with reaction mixture composing of transcription buffer, rNTPs (25 mM ATP, CTP, GTP, and UTP) and enzyme mix. The reaction was incubated at 37 ^{o}C for 4 hours. After that DNA template was eliminated by adding of 1 units of RQ1 (RNase free-DNase enzyme) and incubated at 37 ^{o}C for 15 min. RNA was immediately precipitated using one-tenth volume of 3 M sodium acetate (pH 5.2) and 2 volume of isopropanol. The solution was subsequently incubated at -70 ^{o}C for at least 1 hour and centrifuged at 14,000 g, 4 ^{o}C for 10 min. The supernatant was completely discarded. RNA pellet was washed twice with 1 ml of cold 70% ethanol and resuspended with 100 µl nuclease-free water. Finally, one unit per microlitre of ribonuclease inhibitor (TOYOBO, Japan) was added and long term storage at -70 ^{o}C until use.

3.10 Determination of HIV-1 RNA transcript

Established Avogadro's equation was utilized for copy number determination of *in vitro* transcribed RNA [52-54]. The quantity and quality of RNA was determined

with UV spectrophotometer (OD 260 and 280 nm). The RNA concentration and copy number was calculated by the following equation:

$$\text{RNA concentration } (\mu g/ml) = \frac{\text{OD260 x dilution factor x 40 } \mu g \text{ RNA/ml}}{\text{1 OD 260 unit}} \quad (1)$$

$$\text{Copies/ml} = \frac{\text{NA x RNA concentration (g/ml)}}{\text{(No. of base) x 340 (Da/base)}} \quad (2)$$

Where NA = Avogadro's number (6.023×10^{23} copies/mole)

Da = Daltons

3.11 Real-time PCR for quantitation of HIV-1 RNA

HIV-1 specific primers (HF1, HR1) and probes (HIV-1 specific probe and internal system control probe) were used for quantitation of HIV-1 RNA from plasma samples. One-tube real-time PCR was performed in a total of 20 µl consisting of 10 µl of 2x RNA-direct ^{TM} Real-time PCR Master Mix, 2.5 mM $Mn(OAc)_2$, 0.25 µM TaqMan probes, 0.3 µM each primer and 6 µl of extracted RNA. PCR profiling was conducted into two steps of reverse transcription and cycling amplification. Reaction was initially denatured at 90 ^{o}C for 30 sec and subsequently reverse transcribed at 61 ^{o}C for 20 min. Without false positive in normal human plasma, cycling amplification was accomplished in 48 cycles of denaturation at 95 ^{o}C for 15 sec, annealing at 58 ^{o}C for 20 sec and extension at 72 ^{o}C for 20 sec with final extension at 72 ^{o}C for 5 min. Real-time PCR used for viral quantitation in this study was

Chromo 4™ Real-Time PCR detector (Bio-Rad Laboratories Incorporated. USA). In addition, normal human plasma containing HIV-1 internal system control RNA (approximately 1,000 copies/ml) was used as negative control in every run.

3.12 Optimization of HIV-1 RNA internal system control

To minimize the interference of viral quantitation in individual plasma caused by an internal system control RNA, the latter was 10-fold serially diluted from 10^3-10^7 copies (10 µl), homogeneously mixed with one milliliter of either normal human plasma or normal plasma containing HIV-1 RNA standard (10^3 and 10^4 copies/ml), RNA extraction and analyzed by real-time PCR. In addition, those reactions were compared to the reaction that lacking of an internal system control RNA. A concentration of HIV-1 internal system control that preferred less interference in to the assay was selected and further applied into the standard reaction of real-time PCR.

3.13 Standard curve construction

An external standard curve used for plasma viral quantitation in this study was constructed from HIV-1 *gag* RNA transcript (including an optimized HIV-1 internal system control (10^3 copies/ml). HIV-1 *gag* RNA transcripts were serially 10-fold diluted in normal human plasma from 10^5-10^{10} copies/ml. All plasma samples were extracted and amplified by validated real-time PCR. The standard graph was constructed between the dilution of HIV-1 RNA transcript versus the cycle number.

3.14 The reproducibility of the assay

The reproducibility of validated assay was determined by using an intra-run (within run) and inter-run (different run). HIV-1 *gag* RNA was serial 10-fold diluted in normal human plasma from 10 to 10^6 copies/ml. For intra-run assay, each dilution was examined for six replicates in the same run whereas inter-run assay, each dilution were separately extracted and amplified for eight replicates in different days. Moreover, normal human plasma including with an internal system control (1,000 copies/ml) was applied as negative control in this study. The percent of coefficiency of variation (% CV) was analyzed by using available software.

3.15 Viral RNA quantitation kit used in this study

Based on the Food & Drug Administration approved and routinely employed by CMSU unit, CobasAmpliPrep/CobasTaqman HIV-1 test kit (Roche diagnosticGmbH, USA) was used for virus quantitation in individual plasmas. The results of virus copy number determined by the kit were compared to our validate method.

3.16 Statistical analysis

The correlation and agreement between the developed method and commercial HIV-1 test were analyzed using Pearson Correlation Coefficient and Bland Altman plot, respectively.