CHAPTER IV

MATERIALS AND METHODS

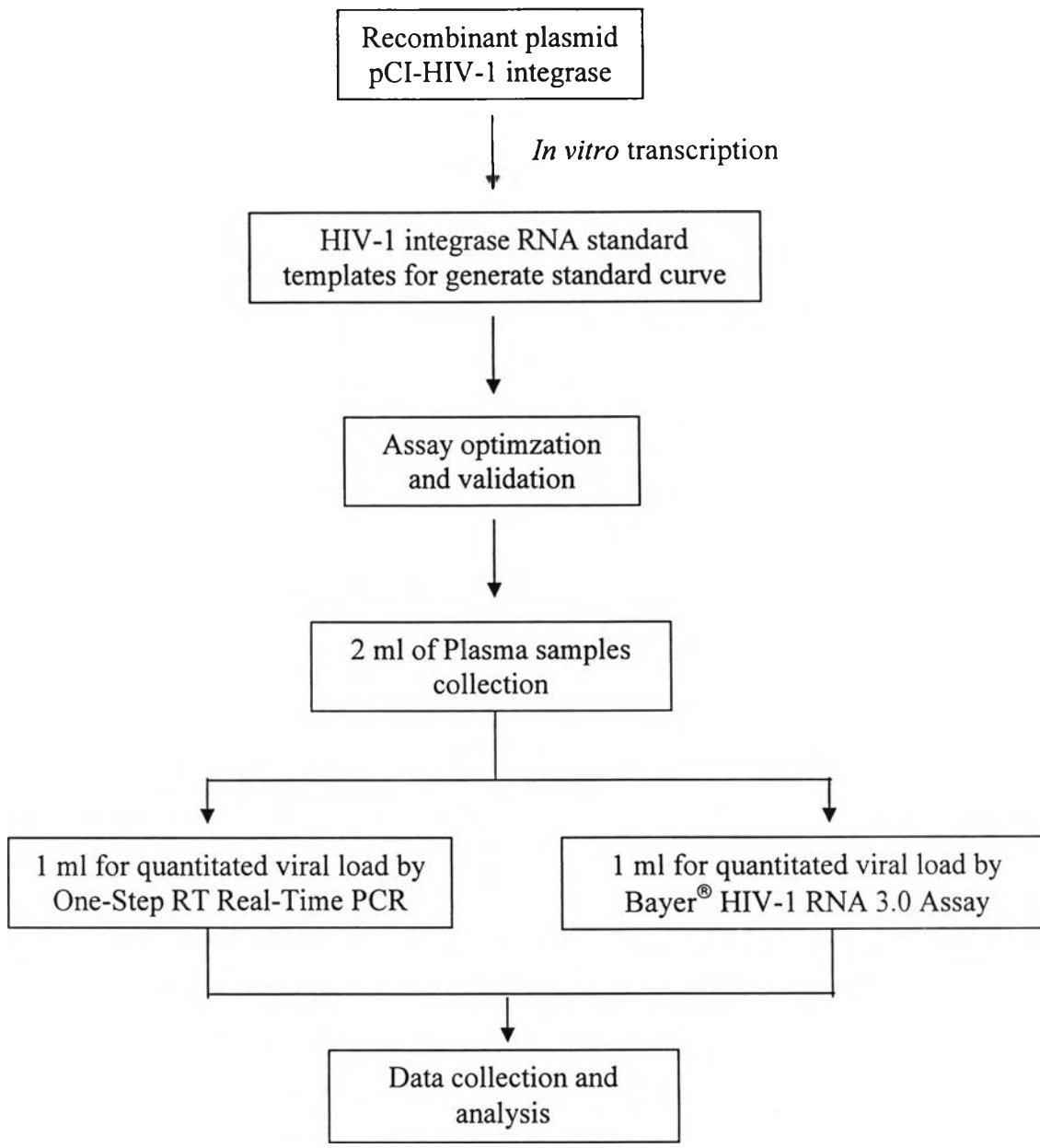

Figure 7 Establishment of quantitation of HIV-1 RNA by in-house One-Step RT Real-Time PCR Assay.

PART I Development of the HIV-1 integrase RNA templates for standard curve.

1.1 Purification of recombinant pCI-HIV-1 integrase cloning plasmid by QIAprep® Miniprep kit

The recombinant pCI-HIV-1 integrase plasmid (provided by Dr. Sunee Sirivichayakul) was propagated and purified with a QIAprep® Miniprep kit according to the manufacturer's reagents and protocol. The pelleted was resuspended and mixed with 250 µl of buffer P1. Then 250 µl of buffer P2 was added to the resuspend pelleted and gently inverted the tube 6 times to mixed. After that buffer N3 of 350 µl was added and gently inverted the tube immediately 6 times. The sample was centrifuged at 13,000 rpm for 10 minutes and applied to a QIAprep column. The column was centrifuged at 13,000 rpm for 1 minute and then washed by centrifuging with 500 µl of buffer PB and 750 µl of buffer PE. The purified pCI-HIV-1 integrase plasmids were eluted with 20 µl of distilled water.

1.2 Quantification of recombinant pCI-HIV-1 integrase plasmid by UV specrophotometry

The recombinant pCI-HIV-1 integrase plasmid was diluted to 1:50 and quantitated by a UV spectrophotometer (Smart Spec™ 3000 spectrophotometer) at A260, A280 and 320 nm. The reading at A260 nm allows calculation of the concentration of nucleic acid in the sample. An OD_{260} of 1 corresponds to 50 µg/ml for double-stranded (ds) DNA, and 40 µg/ml for single-straned (ss) RNA. The ratio between the reading at A260 nm and A280 nm (OD_{260}: OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}:OD_{280} values of 1.8 and 2.0 respectively. If there is significant contamination with protein and phenol, the OD_{260}:OD_{280} will be less than1.8 or 2.0.(82) The OD at 320 nm is used as background and is subtracted from OD_{260} and OD_{280}. Nucleic acid concentrations of dsDNA and RNA are determined by multiplying the difference between the A260 and A320 by the conversion and dilution factors. Nucleic acids absorb strongly at 260 nm so that only a significant level of protein contamination will cause a significant change in the ratio of absorbance at the two wavelengths. (Table 1)

Concentration = (A260 - A320) × conversion factor[a] × dilution factor

[a]OD of 1 corresponds to 50 μg/ml for dsDNA
OD of 1 corresponds to 40 μg/ml for ssRNA

Table 1 Absorbance of nucleic acids and protein.

% protein	% Nucleic acid	OD_{260} : OD_{280}
100	0	0.57
95	5	1.06
90	10	1.32
85	15	1.48
80	20	1.59
75	25	1.67
70	30	1.73
65	35	1.78
60	40	1.81
55	45	1.84
50	50	1.87

% protein	% Nucleic acid	OD_{260} : OD_{280}
45	55	1.89
40	60	1.91
35	65	1.93
30	70	1.94
25	75	1.95
20	80	1.97
15	85	1.98
10	90	1.98
5	95	1.99
0	100	2.0

Note that estimates of purity of nucleic acids based on OD_{260}:OD_{280} ratios are accurate only when the preparations are free of phenol.(82)

1.3 Preparation of linearized recombinant pCI-HIV-1 integrase plasmid templates.

Recombinant pCI-HIV-1 integrase cloning plasmid templates were linearized by restriction endonuclease. The restriction conditions: 15 μg of pCI-HIV-1 integrase DNA templates, 2.5 μl of 10xSuRE/cut BuffA and 45 U of *Sma*I (Roche, Penzberg, Germany). The solution was incubated at 25 °C overnight. A QIAquick spin column (QIAGEN, Hilden, Germany) was used to purify the linearized pCI-HIV-1 integrase DNA templates according to the manufacture's protocol. Buffer PB was added in a ratio of 1:5 to the solution and mixed. The sample was applied to a QIAquick column and centrifuged at

13,000 rpm for 1 minute. The column was washed by centrifugation with 750 µl of buffer PE and the purified linearized pCI-HIV-1 integrase DNA templates were eluted with 20 µl of distilled water.

1.4 Preparation of HIV-1 integrase RNA templates preparation by *in vitro* transcription system.

HIV-1 integrase RNA was prepared by RiboMAT™ Large Scale RNA Production System–SP6 and T7 (Promega Biosciences, INC. San Luis Obispo, CA). Optimal RNA yields depend on starting with a high-quality DNA templates (OD_{260}:OD_{280} value between 1.8-2.0) and yields of linearized DNA suitable for transcription reactions. It is important that no RNase is present in the linearized DNA template.

The reaction components for synthesis of HIV-1 integrase RNA templates were added in the order shown in Table 2. The purified linearized pCI-HIV-1 integrase DNA templates were carefully dissolved in nuclease-free water before addition to the reaction. The HIV-1 integrase RNA solution was gently pipetted to mix and incubated at 37 °C for 3 hours. Remaining linearized pCI-HIV-1 integrase DNA templates were removed by adding Promega's RQ1 RNase-free DNase to a concentration of 1U/µl and incubating at 37 °C for 6 hours.

Table 2 T7 reaction Component *In vitro* transcription system

T7 Reaction Component	Sample Reaction	Control Reaction
T7 Transcription 5x Buffer	20 µl	4 µl
rNTPs(25mM ATP, CTP, GTP, UTP)	30 µl	6 µl
linear DNA template (5-10 µg total)		(control DNA) 1 µl
plus Nuclease-Free water	40 µl	(water) 7 µl
Enzyme Mix (T7)	10 µl	2 µl
Final volume	100 µl	20 µl

1.5 Purification of RNA by phenol: chloroform extraction.

The HIV-1 integrase RNA templates were purified with phenol: chloroform extraction by adding 1 volume of TE-saturated (pH4.5) phenol: chloroform: isoamylalcohol (25:24:1) and mixing well by vortexing for 1 minute. The HIV-1 integrase RNA solution was centrifuged at 13,000 rpm for 2 minutes. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform: isoamyl alcohol (24:1) was added. Next the solution was centrifuged at 13,000 rpm for 1 minute. At this point, unincorporated nucleotides may be precipitated directly. The upper aqueous phase was transfer to a fresh tube and any containing chloroform was removed by performing a quick spin (10 second) at 13,000 rpm followed by removal of the bottom phase with a micropipette. 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanal were added to the solution, mixed and placed on ice for 5 minutes. The HIV-1 integrase RNA was centrifuged at 13,000 rpm for 10 minutes and the supernatant was carefully poured. The pellet was washed with 1 ml of 70% ethanol and dry under vacuum at room temperature for 20 minutes. The purified HIV-1 integrase RNA templates were resuspended with HIV-1 diluents (Roche Molecular System, Inc. Branchburg, NJ, USA.) and the concentration was determined by RiboGreen™ Quantitation kit (Molecular Probes) measurement.

RiboGreen is a RNA specific binding dye. With a detection range of 2 ng to 1 μg of RNA per ml, a much higher sensitivity and a broader measurement range than can be obtained with UV absorbance measurement. Also electrophoresis was performed to determine the quality of the purified HIV-1 integrase RNA constructs. Complete DNase digestion was tested by PCR (without the reverse transcription step). The purified HIV-1 integrase RNA constructs were aliquoted for single use and stored at -70 °C.

1.6 RNA electrophoresis to determine the quality of RNA

HIV-1 integrase RNA templates were analyzed by RNase-free agarose gel electrophoresis. DIETHYL PYROCARBONATE (DEPC)-treated RNA sample buffer was added in a ratio of 1:1 to the HIV-1 integrase RNA templates. The RNA sample was heated at 60 °C for 5 minutes and then cooled on ice for 2 minutes. The RNA sample was added to 2 μl of DEPC-treated RNA loading buffer (Bromophenol blue indicator) and 200 ng of ethidium bromide and then applied to the wells of 2% RNase-free agarose gel in DEPC-

treated 1X MOPS buffer. RNA markers (Promega) were included. A voltage of 60 V was applied for 40 minutes. The gel was visualized using a UV light transilluminator. The image was captured with a Polaroid-camera with black and white print film (Sigma). The size of the RNA product band was read directly from the gel electrophoresis as shown in Figure 8.

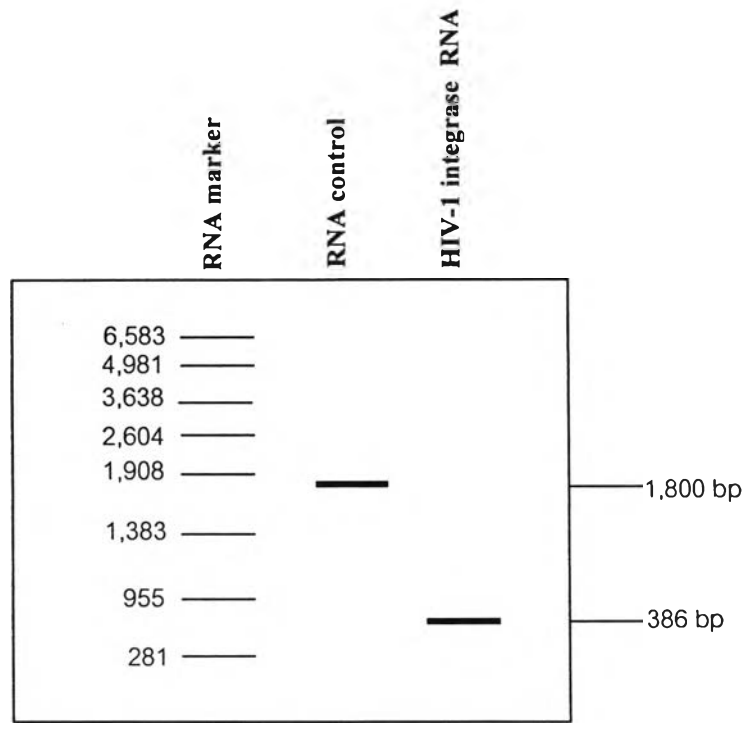

Figure 8 Diagram for determining the size of the RNA product band directly in the gel electrophoresis.

PART II Development of One-Step RT Real-Time PCR for Quantification of HIV-1 RNA.

2.1 PCR primer and labeled TaqMan probe Design

In order to avoid major mismatches due to HIV-1 variability, the primers and probe were designed based on the published sequences in the Los Alamos National Laboratory Database using Oligo software. Primers are located in the highly conserved region within the integrase gene of HIV-1 with a melting temperature (Tm) of 58–60 ^{o}C and both primers have an equal for Tm. Primers should be 15-30 bases in length and the G+C content should ideally be 30-80%. The total number of Gs and Cs in the last five nucleotides at the 3'end of the primer should not exceed two. This helps to introduce relative instability to the 3'

end of primer to reduce non-specific priming. Maximum amplicon size should not exceed 400 bp (ideally 50-150 bp). In our case, the amplified product is 111 bp in length. The forward primer sequence is 5'-CGTTGTCTGTATGTTTCATTTG-3' and the reverse primer is 5'-CCTTTCCTGGTCGTTTTGAG-3'(Table 3). The TaqMan probe sequence was selected based on (i) G/C rich regions. (ii) melting temperature of 68-70 °C (iii) no stretches of identical nucleotides longer than four (iv) no G at 5' end due to quenching activities of this nucleotide and (v) 10 °C higher melting temperature than the primers. The nucleotide sequence of the probe is 5'R-AAGCCCAAATAATGAGGGTGACG-Q3'(Tabel 4), where R indicates the reporter dye 6-carboxy fluorescein (FAM) and Q indicates the quencher dye 6- carboxy-tetramethyl-rhododamine (TAMRA) (Tabel IV).

Table 3 Sequence of primer and size of PCR products specific for HIV-1 integrase (subtype A/E)

Target	Product size (bases)	Primer sequence
INT INT_4416 INT_4527	111 bp	5'-CGTTGTCTGTATGTTTCATTTG-3' 5'-CCTTTCCTGGTCGTTTTGAG-3'

Table 4 Sequence of TaqMan probe specific for HIV-1 integrase (subtype A/E)

Target	Probe sequence	Fluorescein
INT_P	5'R-AAGCCCAAATAATGAGGGTGACG-Q3'	6-carboxy fluorescein (FAM): R 6-carboxy-tetramethyl-rhododamine (TAMRA) : Q

2.2 Optimization of One-Step RT Real-time PCR Assay

Primers and TaqMan probes for One-Step RT Real-Time PCR are designed base on the 5' ⟶ 3' nuclease activity of DNA polymerase. The dually labeled probes that specifically hybridize to the target sequence between the two primers are cleaved off by the polymerase during the primer extension phase of PCR. The reporter fluorescence (previously quenched by the proximity of the quencher dye) is then emitted. The elapsed time (or number of cycles of PCR) until the reporter fluorescence rises over a certain threshold is inversely correlated with the amount of target sequence present in the sample. The ABI PRISM 7700 sequence detection system (PE Biosystems, Foster City, CA) converts this time point into the so called threshold cycle (C_t) for each sample. The lower the C_t, the higher the target input amount is. The annealing temperature at 48, 50, 55, 60 °C and also probe concentration at 66, 100, 200 nM and primer concentration at 100, 200, 300 nM will be evaluated. The most optimized with cost effective is shown Table 5. The One-Step RT-PCR Master Mix kit (PE Applied Biosystem cat # D03879) will be run under the following condition: 25 µl of 2X PCR Master Mix without UNG, 1.25 µl of 40x MultiScribe and RNase Inhibitor Mix, 300 nM of each forward and reverse primers, 66 nM of probe and 20 of 60 µl of HIV RNA eluted. Cycling condition is 30 min at 48 °C for reverse transcription, 95 °C 10 min for AmpliTaq Gold Activity, followed by 45 cycles of amplification (95 °C for 15 sec, 60 °C for 1 min).

Table 5 Condition of One-Step RT Real-time PCR Assay

Primer	Probe	Real time PCR conditions	Cycling condition
INT_4416 INT_4527	INT_P	25 µl of 2X PCR master Mix 300 nM of primer INT_1 300 nM of primer INT_2 66 nM of probe INT_P 1.25 µl of 40x MultiScribe and RNase Inhibitor Mix 20 of 60 µl of HIV RNA eluted	ABI7700 real time PCR system Reverse transcription at 48°C for 30 min. Activate AmpliTaqGlod at 95°C for 10 min 45 cycles of 95 °C for 15 sec 60 °C for 1min

2.3 Analysis of One-Step RT Real-Time PCR Assay.

The linear range of assay will be prepared as 5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 RNA copies/ml of HIV-1 integrase RNA templates. The ABI PRIMS 7700 is able to give a threshold cycle (C_t) value for every sample. The C_t value allows the quantitation of input RNA by comparison with the C_t of standard RNA.

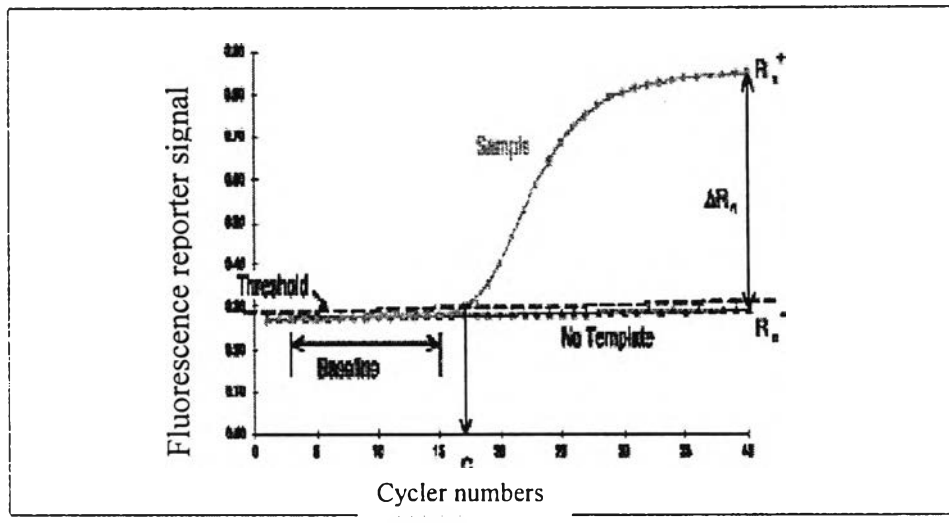

Figure 9 Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR (figure from Applied Biosystem). The graph of the increment of fluorescence reporter signal ΔRn versus cycle number during PCR shows three stages: baseline, exponential phase, and plateau. The C_t value is calculated by determining the point at which the fluorescence exceeds an arbitrary threshold limit.

The concept of the threshold cycle (C_t) is at the heart of accurate and reproducible quantification using fluorescence-based RT-PCR(83). Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background(84). This point is defined as the C_t, and will always occur during the exponential phase of amplification.

PART III Validation of the assay

3.1 Specificity of the One-Step RT Real-Time PCR for quantitating HIV-1 RNA

The specificity of the assay was determined by testing plasma obtained from 25 HIV-negative blood donors by using One-Step RT Real-Time PCR as described in 2.2 procedure. The recombinant pCI-HIV-1integrase plasmid (as described in 1.1) was used as a positive control and distilled water was used as a negative control.

3.2 The efficiency and sensitivity of the One-Step RT Real-Time PCR for quantitating HIV-1 RNA.

The efficiency and the sensitivity of assay were determined by testing the HIV-1 integrase RNA standard templates. The HIV-1 integrase RNA templates were diluted in 45 µl of HIV-1 diluents to obtain 5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 copies/ml. The diluted HIV-1 integrase RNA templates were treated as described in 2.2 procedure. The recombinant pCI-HIV-1 integrase plasmid (as described in 1.1) was used as a positive control and distilled water was used as a negative control.

3.3 The intra-assay and inter-assay reproducibility and linearity of the One-Step RT Real-Time PCR for quantitating HIV-1 RNA

The intra-assay variation was determined by testing each dilution of 10 replicates of HIV-1 integrase RNA template (5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 copies/ml) in the same experiment. The inter-assay variation was determined by using 5 different dilutions of HIV-1 integrase RNA template (5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 copies/ml) each repeated 5 times independent experiment. The reproducibility of RNA extraction procedure was analyzed by using 4 replicates of 5 different dilutions of HIV-1_{IIIB} viral particles (5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 copies/ml) added to HIV-negative plasma to mimic the patient samples. This assay was repeated 2 times. The percent of coefficiency variation was determined by http://faculty.vassar.edu/lowry/VassarStats.html website for assessing the reproducibility. The recombinant pCI-HIV-1integrase plasmid (as described in 1.1) was used as a positive control and distilled water was used as a negative control.

PART IV HIV-1 quantitated in HIV-1 infected patients: Comparative of plasma HIV-1 quantitation using Bayer® HIV-1 RNA 3.0 Assay (bDNA) versus One-Step RT Real-Time PCR assay in HIV-1 seropositive patients.

4.1 Study group.

One hundred randomized of HIV-1 infected individuals from the Anonymous Clinic, Thai Red Cross AIDS Research center were recruited into this study by basis of the following inclusion criteria:

The HIV-1 infected condition is verified by anti-HIV positive of 2 anti-HIV antibody screening assays with different test principle (*i.e.*) anti-HIV Rapid Test Double Check Test and Gel particle Agglutination.

This study protocol was approved by the Human Ethical Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from all study patients.

4.2 Specimen collection

Seven milliliters of blood were collected from each subject by venepuncture into EDTA tubes. Plasma was separated by centrifugation at 3,000 rpm for 10 minutes within 3 hours after collection. The plasma samples were aliquoted to 1 milliliter and immediately frozen at -70 °C until they were ready for processing and analysis.

4.3 Plasma HIV-1 RNA preparation for One-Step RT Real-Time PCR analysis.

HIV-1 RNA was purified with a QIAamp® Viral RNA Mini kit according to manufacturer's protocol. One milliliter EDTA plasma was centrifuged at 13,000 rpm for 1.30 hour at 4 °C. 860 µl of supernatant was carefully discarded. Viral RNA was extracted from the viral pellet by adding 560 µl of AVL buffer and incubating for 10 minutes at room temperature. The RNA was precipitated with 560 µl of 100% ethanol and then the solution was applied to a QIAamp spin column and centrifuged at 8,000 rpm for 1 min at 25 °C. The QIAamp spin column was washed with 500 µl of AW1 and AW2. The HIV-1 RNA was resuspended in 60 µl of nuclease free water and centrifuge at 8,000 rpm for 1 minute. Isolated RNA was stored immediately at -70 °C until amplification.

4.4 cDNA synthesis

Reverse transcriptase reaction was carried out as a single tube reaction with uninterrupted thermal cycling by using the GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Conn.). A reaction solution was prepared and a 20 µl was aliquot in a thin-walled MicroAmp tube. The final reaction mixture contained 10 µl of RNA sample, 300 nM of primer INT_4527, 1X RT-buffer, 5mM $MgCl_2$ and 2 mM dNTPs. The solution was added to 100 U of recombinant Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase and 80 U of RNase Inhibitor (Promega). The reaction was incubated at 42 °C for 30 minutes. cDNA sample was stored at -20 °C until amplification.

4.5 PCR amplification

Polymerase chain reaction was carried out as a single tube reaction with uninterrupted thermal cycling by using the GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Conn., USA). A reaction solution was prepared and a 20 µl aliquot was in a thin-walled MicroAmp tube. The final reaction mixture contained 1X PCR-buffer, 2 mM $MgCl_2$, 0.8 mM each dNTPs, 300 nM of each primer and 1.25 Units *Taq* polymerase (Promega). In a volume of 30 µl [by addition of nuclease-free water (Gibco/BRL)] and 20 µl of cDNA. The PCR conditions include pre-heat at 94°C for 5 minutes, then 40 cycles of 1 minute at 94 °C, 1 minute at 60 °C and 1 minuite at 72 °C, with a final extension step at 72 °C for 7 minutes.

4.6 Analysis of amplification products

PCR products were analyzed by agarose gel electrophoresis. 15 µl of PCR product was mixed with 3 µl of 6X loading buffer (bromphenol blue indicator) and applied to the wells of a 1.8% agarose gel with 0.3 mg/ml ethidium bromide in Tris-borate-EDTA-buffer. A 100 bp DNA marker ladder (Bio-Rad) was included. A voltage of 80 V was applied for 20 minutes and then the gel was visualized using a UV light transilluminator. The image was captured with a Polaroid-camera with black and white print film (Sigma). The PCR product band size was read directly from the gel (Figure 10).

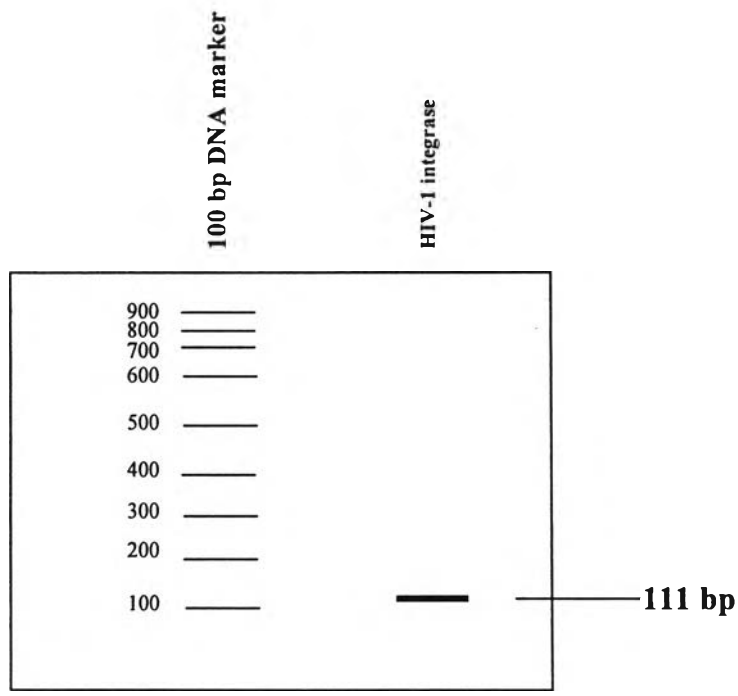

Figure 10 Diagram for the 111 bp of RT-PCR product patterns after agarose gel electrophoresis

4.7 Comparison of the One-Step RT Real-Time PCR Assay for quantitation of HIV-1 RNA and Bayer® HIV-1 RNA 3.0 Assay (bDNA) [Bayer, Emeryville, CA].

For a large scale validation, HIV-1 RNA of one hundred plasma samples were quantitated by One-Step RT Real-Time PCR and compared to Bayer® HIV-1 RNA 3.0 Assay (bDNA) [Bayer, Emeryville, CA]. All the HIV EDTA-plasma from HIV-1 infected individuals were collected and aliquoted into 2 tubes of 1 ml each and frozen at – 70 °C. One aliquot was quantitated with the commercial Bayer® HIV-1 RNA 3.0 Assay (bDNA). The other one was quantitated with the RT real-time PCR assay. The correlation of both assays were analyzed by Pearson correlation coefficient (*r* value), Wilcoxon Signed Ranks Test by using the SPSS program and Bland Altman plot by using GraphPad Prism program.