CHAPTER III

MATERIALS AND METHODS

1. Matherials

1. 1 Plasma samples

1.1.1 HIV-1 positive pool plasma

HIV-1 positive plasmas with viral load more than 100,000 copies/ml were pooled and distributed into 1 ml aliquots and keeping at -70ºC. thre e aliquots were sampling for determination of HIV-1 viral load by using Amplicor HIV-1 Monitor® reagent kit, version 1.5 (Roche Molecular System Inc., USA). The mean of viral load were calculated and used for construction of RNA standard curve.

1.1.2 HIV-1 negative plasma

The HIV-1 negative plasmas were collected and pooled from expired donor's blood from Blood Banking Unit at Maharaj Nakorn Chiang Mai Hospital. All plasmas were identified as HIV-1 serological negative according to the WHO criteria.

1.1.3 Patient samples

Five milliliters of blood samples were drawn and collected in EDTA anticoagulant tubes (Becton Dickinson Inc., USA) Plasma was separated by centrifuge at 1,200 × g, for 20 min and collected in 2 aliquots of 1 ml in two-ml screwed-cap tubes (SARSTEDT, Germany) and kept at -70°C. One of plasma aliquot was sent for routinely tested of HIV-1 viral load at Clinical Service Center, Faculty of Associated Medical Sciences, Chiang Mai University using the Amplicor HIV-1 Monitor® reagent kit, version 1.5. The different groups of viral load results, very high (> 750,000 copies/ml), high (<750,000-100,000 copies/ml),

moderate (<100,000-10,000 copies/ml), low (<10,000-1,000 copies/ml) and not detected (<400 copies/ml) copy number were randomly collected and used for validation of the in-house assay developed in this study.

1.2 PCR primers and the TaqMan probe design

HIV-1 specific primers and the TaqMan probe used in this study were previously designed and kindly provided by the group of Dr. Pranee Leechanachai, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Their nucleotide sequences and location were shown in Table 4 and figure 3. Both oligonucleotide primers and probe were designed to amplify the *gag* sequence of HIV-1 subtype AE. However, the specificity of the designed primers and probe were finally tested with all (545 sequences) HIV-1 AE and B *gag* sequences shown in GenBank data base using computer solfware (BLASTn; Basic Local Alignment Search Tool of nucleotide) before synthesizing and used for further experiments.. The result showed that they were specific to all sequences tested. The synthesized primers can also amplify HIV-1 RNA when tested with various HIV-1 positive plasma samples.

The TaqMan probe which designed following the general rules outlined by the manufacturer was labeled with 6-carboxy fluorescein FAM (6-FAM) as a reporter dye at the 5' end, and a quencher dye, 6-carboxy tetramethyl rhodamine (TAMRA) at the 3' end (Operron BiotechnologiesGmbh,Germany)(Figure 3).

As long as both fluorochromes were on the probe, the quencher molecule stoped all fluorescence by the reporter. The probe was designed to have a higher Tm than the primers, and during the extension phase, the probe must be 100% hybridized.

Figure 3 Relative locations of HIV-1 specific primers and HIV-1 probe on the HIV-1 *gag* gene.

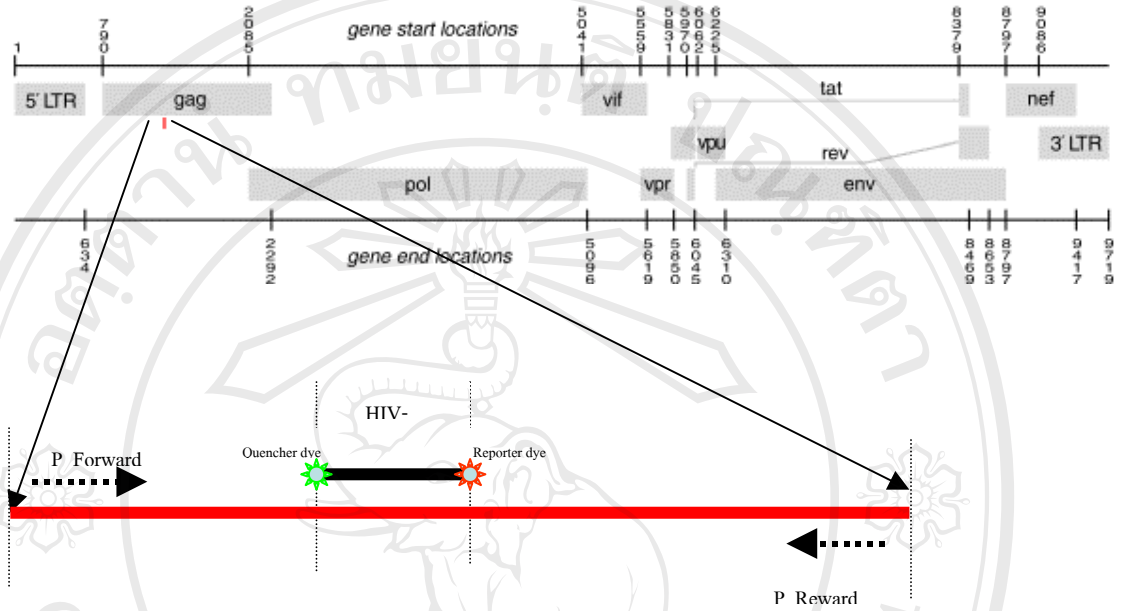

Table 4 Sequences of the primers and probe used for in-house real-time RT-PCR

Primers	Tm	GC%	Length	Sequences (5'-3')
P_FWD	57.2	45.5	22	TAC CCG TGT TCT CAG CAT TAT C
P_RWD	57.1	30.4	23	GAT GGT TTC TTT TAA CAT TTG CA
HIV_P	67.1	39.3	28	AGC CAC CCC ACA AGA TTT AAA TAT GAT G

P_FWD, forward primer; P_RWD, reward primer; HIV_P, HIV-1 probe, Tm; melting temperature, GC%; percentage of GC content

1.3 Reagents for nucleic acid purification

QIAmp® Viral RNA Mini Kit (Qiagen, Germany) composed of all reagents (lysis , binding and elution buffer) for extraction of viral RNA from plasma or cell free body fluid. The kit combines the selective binding properties of silica gel-based membrane with the speed of microspin technology and is suited for simultaneous processing of multiple samples.

1.4 Reagents for real-time RT-PCR

Quantitect™Probe RT-PCR kit (Qiagen, Germany) is a reagent mix for reverse transcriptase PCR on the real-time PCR format. The reagent composed of hot-start enzyme (HotStarTaq DNA polymerase) in order to ensure the specificity of reaction and Omniscript and Sensiscript reverse transcriptase to enhance the sensitivity. All PCR components have been optimized for use with any real-time cycler including LightCycler™.

2. Methods

2.1 HIV-1 RNA extraction

HIV-1 RNA was extracted from plasma by using the QIAmp® Viral RNA Mini Kit (Qiagen Gmbh, Germany) following the manufacturers' instructions. Briefly, first, RNA extraction was began with the lysis of viral particles in 140 µl of plasma under highly denaturing conditions, inactivating RNAses and ensuring isolation of intact RNA. Poly A carrier RNA in the 560 µl AVL lysis buffer enhanced RNA recovery in subsequent steps. Next, buffering conditions were adjusted with 560 µl absolute ethanol to provide optimum binding to a silica gel based capture membrane held the QIAamp spin column. The sample was loaded onto the spin column and the RNA bind to the silica gel membrane. Contaminants were washed away with two different wash buffers. After the wash buffers had passed through the column, purified RNA was eluted with 50 µl of an RNase free elution buffer. The extracted RNA was stored at -70 °C until use.

2.2 One-step real-time RT-PCR on LightCycler™

The RT-PCR was carried out using 8 ul of RNA template extracted from HIV-1 positive plasma in a final reaction volume of 20 ul. The assay was performed by using the Quantitect™ Probe RT-PCR kit (Qiagen) and prepared following the manufacturers' instructions shown in table 5. The final concentrations in reaction mixture were 4 mM $MgCl_2$, 1 mM dNTP, 1 µM of each ologonucleotide primer and unknown of RT Mix (containing Omniscript and Sensiscript reverse transcriptase). The amplification was carried out as follows: reverse transcription at 50°C for 20 minutes, initiation activation of Hot start enzyme at 95°C for 15 minutes, 50 cycles of three-step PCR cycle (0 s at 95°C, 5 min 58ºC and 20 s at 60°C) and finally cooling at 4º C.

Concentration of the key components such as $MgCl_2$, primers and probe were previously optimized by Ms. Thanawan Sumleerat (data not shown).

Table 5 The components of one-step RT-PCR reaction

Components	Final concentraation	µl/Rx (20 µl)
RNase free water	-	1.2
2X QuantiTech Probe RT-PCR Master Mix	1x	10
100 pmol/µl FWD Primer	1.0 µM	0.2
100 pmol/µl RWD Primer	1.0 µM	0.2
20 pmol/µl HIV_ Probe	0.2 µM	0.2
100X QuantiTect RT Mix enzyme	NK	0.2
RNA Template		**8**

NK; not known

2.3 HIV-1 RNA Standard quantitation curve

Pooled HIV-1 positive plasma were diluted in 10-fold serial dilution from $1x10^5$ to $1x10^2$ copies/140 µl and processed for RNA extraction and RT-PCR on LightCycler™ system. Each dilution was run in duplicate. The fluorescent signal obtained from each dilution was plotted against the cycle number at which fluorescence raises above the baseline level call threshold cycle (C_T). Then a regression curve was plotted between C_T and $\log_{10}$ concentration of RNA (copy number).

2.4 Quantitation of HIV-1RNA from plasma samples

One hundred forty microliters of plasma was extracted for RNA and 8 µl was used for amplification by RT-PCR on the LightCycler™ system. Analysis of the amplified RNA was carried out by LightCycler™ software version 3.5 (Roche). The software determines the C_T that is proportional directly to the $\log_{10}$ of the copy number of the input template with respect to a standard curve generated in pararelled. The result was calculated and reported in copy per milliliter of plasma.

2.5 Reproducibility and precision studies

The study was designed to evaluate the variability between different runs (inter-run) and the same runs (intra-run) of HIV-1 viral load assay using our method, an in-house real-time RT-PCR.

HIV-1 positive pool plasma (3.98×10^6 copies/ml) was serially diluted with negative pool plasma to viral load ranging from 10^2 to 10^5 copies/ml. For inter-run assay, each dilution of plasma was processed for RNA extraction and amplification in duplicate. Each assay was repeated 3 times. The intra-run assay was performed with five replicate of each plasma dilution and assay at the same time. The results were analyzed by comparing mean, S.D. and %C.V. of C_T value of each run.

2.6 Lower limit of detection

HIV-1 positive pool plasma was diluted to 1,000, 500, 100, and 50 copies/ml with HIV-1 negative pool plasma and aliquots 500 µl of each dilutions for ten aliquots and kept at -70ºC (Table 6). The assay was run for ten times in all

dilutions and calculated in copy number/ml with respect to standard curve. The lowest HIV-1 RNA copy number detected by the assay was implied as lower detection limit

Table 6 Dilutions of pool plasma standard for lower limit detection experiment

	Concentration (copies/ml)						
	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1	5×10^2	5×10^1
Pool plasma stock (3.89×10^5 copies/ml)	100	-	-	-	-	-	
HIV-1 negative plasma (µl)	289	1800	5,400	5,400	5,400	3,000	5,400
	200	**600**	**600**	**600**		**600**	
				3,000			

2.7 Comparative cost analysis.

For comparative analysis of costs of disposables and labor, the routine assay procedures were followed for the in-house RT-PCR was described before and the Amplicor HIV-1 Monitor® test (version. 1.5) assays, as described in the package inserts. Costs for disposables were based on list prices as of October 2005 from the vendors in Thailand. No discounted pricing was used. For the assessment of labor, each procedure was timed from the start to the end for one sample.

Costs of disposables for the Amplicor HIV-1 Monitor® test (version. 1.5) assay were determined for each of the three workflow steps. Costs of sample preparation disposables were calculated for the following: 3 aerosolized 200-µl tips (4,494 bath/960), 6 aerosolized 1,000-µl tips (3,424 bath/960), 1,000 µl of reagent grade ethanol (890 bath/2.5 liters) and 800 µl of isopropanol (548 bath/2.5 liters)**.** And, cost of disposables for amplification and detection were calculated for the following: 9 aerosolized 200-µl tips (4,494 bath/960).

Costs of disposables for the in-house assay were determined for each of the two workflow steps. Costs of sample preparation disposables were calculated for the following: 1 aerosolized 200-µl tips (4,494 bath/960), 6 aerosolized 1,000-µl tips (3,424 bath/960), 1 aerosolized 10-20 µl tips (5,800 bath/960) and 560 µl of reagent grade ethanol (890 bath/2.5). And cost of disposables for amplification and detection was 7 aerosolized 20-µl tips.