QUANTITATIVE DETECTION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) VIRAL LOAD BY REAL-TIME RT-PCR ASSAY USING SELF-QUENCHED FLUOROGENIC PRIMERS

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Abstract. HIV-1 viral load is a basic marker to evaluate the severity of HIV-1 related diseases and to monitor the effectiveness of treatment. A method based on real-time RT-PCR technology has been developed to quantify HIV-1 RNA using self-quenched fluorogenic primers known as LUX™ primers. They were used in this study to recognize a low variable *gag* region of subtype E and B consensus sequences. Specificity was verified by amplicon melting temperatures. An external standard curve was constructed with 10 fold serial dilutions of synthetic HIV-gag RNA. A broad range linear relationship (10 to 10^6 copies/ml) was observed between the number of PCR cycles needed to detect a fluorescent signal and the number of RNA copies. The intra- and inter-assay coefficients of variation were 0.72 to 2.54% and 3.14 to 8.83%, respectively, thus indicating good reproducibility. Thirty out of fifty HIV-infected individual plasma samples were quantified by this method and compared with the AMPLICOR® HIV-1 Monitor assay, which is widely considered the reference technique for HIV-RNA viral load measurement. The results indicate that the AMPLICOR® HIV-1 Monitor assay and real-time RT-PCR using LUX™ primers are in good agreement (mean difference in log10 copies/ml ± 2 standard deviations = 0.21 ± 1.34).

INTRODUCTION

The measurement of human immunodeficiency virus type 1 (HIV-1) RNA levels in plasma (viral load) is one of the most valuable clinical tools for predicting disease progression (Coombs, 1994, 1996; Cao *et al*, 1995; Mellors *et al*, 1995; O'Brien *et al*, 1996), determining the need to initiate or monitor antiretroviral therapy (Bagnarelli *et al*, 1995; Schooley, 1995; O'Brien *et al*, 1996; Saag *et al*, 1996), and evaluate the efficacy of newly developed antiretroviral drugs (Schooley, 1995). Several commercial assays, employing different molecular technologies to measure plasma HIV-1 RNA levels, are available. These methods include reverse transcriptase (RT) PCR, in the AMPLICOR HIV-1 Monitor assay (Roche Diagnostic Systems, USA) (Mulder *et al*, 1994), nucleic acid sequence-based amplification or NASBA, in the NucliSens HIV-1 RNA QT assay (Organon-Teknika, Boxtel, The Netherlands) (Kievits *et al*, 1991; van Gemen *et al*, 1994, 1995), and branched-DNA (bDNA) techniques, in the Quantiplex HIV RNA assay (Chiron Corporation, USA) (Pachl *et al*, 1995).

The recent introduction of several real-time detection systems were a big step forward in the quantification of viral load, because these easy to-use platforms to detect and quantify viral RNA and DNA targets over a large dynamic range can accommodate at least eight log10 copies of nucleic acid template (Ishiguro *et al*, 1995; Abe *et al*, 1999; Kimura *et al*, 1999; Ryncarz *et al*, 1999; Locatelli *et al*, 2000; Najioullah *et al*, 2001). Real-time PCR is also an attractive alternative to conventional PCR for the study of viral load because of its low inter- and intra-assay variability (Abe *et al*, 1999; Locatelli *et al*, 2000; Schutten *et al*, 2000) and its equal

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or greater analytical sensitivity compared to traditional viral culture, and conventional single-round or nested PCR (Lanciotti *et al*, 2000; Locatelli *et al*, 2000; Kearns *et al*, 2001; van Elden *et al*, 2001). The turnaround time necessary for the generation of results should be short enough to enable therapeutic intervention. The hands-on time for the entire process of nucleic acid isolation, amplification, and quantitative detection from a clinical sample should be minimal. Automation of any step involved in the process is critical.

We have developed a low cost, in-house HIV-1 quantification assay using real-time RT-PCR technology using a LightCycler® system with self-quenched fluorogenic primers (LUX™ primers) as the detectors. The LUX™ (Light Upon eXtension) fluorogenic primers are PCR primer sets in which one of the two primers is labeled with a single fluorophore close to the 3′ end (Nazarenko *et al*, 2002a,b; Bedecarrats *et al*, 2003; Lowe *et al*, 2003). Recently published articles showed that LUX™ Primers have been shown to routinely detect 100 or fewer copies of a target gene and measure picogram amounts of DNA and RNA (Nazarenko *et al*, 2002a,b; Bedecarrats *et al*, 2003; Lowe *et al*, 2003). They support melting curve analysis and multiplexing using different fluorophorelabels, and are compatible, with a variety of real-time instruments. In addition, this innovative technology platform provides sensitivity, specificity, and cost-effective real-time detection without dual-labeled probes or DNA binding dyes. The target sequences for the RT-PCR reaction were chosen in a conserved-gag region of subtypes E and B that are 2 major HIV-1 subtypes found in Thailand (Ou *et al*, 1993). Fluorescence of the LUX™ primer was monitored at a single specific point during each amplification cycle. This allows the determination of initial RNA viral copies by comparison with an RNA standard curve produced by *in vitro* transcription and quantified by an ultrasensitive fluorescent nucleic acid stain (Jones *et al*, 1998), RiboGreen® RNA quantitation dye, which has been widely used in research carried out regarding *in vitro* transcription technology (Muriaux *et al*, 2001; Cheung *et al*, 2004; Kuhlman *et al*, 2004). The real-time RT-PCR assay was used to evaluate HIV infected plasma and compared to the log10 viral load of the Amplicor Monitor assay (copies/ml), to assess a quantitative relationship.

MATERIALS AND METHODS

Samples

Fifty HIV-1 infected-patient plasma samples were obtained from patients tested for HIV-1 viral load at our laboratory. There were twenty-three subjects with a viral load of ≥100,000 copies/ml determined by AMPLICOR® HIV-1 assay. The rest had viral loads ranging from 400 to 100,000 copies/ml. Other RNA virus infected-patient samples (hepatitis C virus, influenza virus, and dengue virus types 1-3) were used to test the specificity of the Real-time RT-PCR method. HIV-1 cultured in 1A2 cells, which secrete HIV-1 proviral mutants functionally defective in *tat* and *rev*(VIII $B^{\Delta tat/rev}$), was used as a positive control. Normal human plasma and water were used as negative controls.

RT-PCR HIV-1 viral load determination (AMPLICOR® HIV-1 Monitor assay)

All HIV-1 positive plasmas were extracted and quantified by the AMPLICOR® HIV-1 monitor kit v1.5 (Roche, USA) using COBAS AMPLICOR analyzer following the manufacturer's instructions. The amount of HIV-1 RNA was expressed as the number of copies per milliliter of plasma. The lower detection limit of the standard method was determined at 400 copies/ml.

RNA extraction and purification from plasma

Whole blood samples were collected from RNA virus infected patients (HIV-1, hepatitis C virus, influenza virus, dengue virus types 1-3, and enterovirus) by venipuncture in EDTA-containing tubes. The samples were centrifuged at 2,500 rpm for 20 miniutes and the plasma was stored at -80ºC until use. In order to extract and purify the RNA viral genome, 140 μl of RNA virus infected patient's plasma was processed with a QIAamp® Viral RNA Mini Kit (QIAGEN, USA) following the manufacturer's instructions. Purified RNA was eluted in 50 μl of kit elution buffer.

Primer design

Two sets of primers were designed and

used in this study. The first set of primers was designed to amplify the gag-region of HIV-1 RNA using Primer Premier 5 software based on the gag-region of HIV-1 Consensus_M 2002 sequence (retrieved from http://hiv-web.lanl.gov). The amplified products derived by this set of primers were cloned into a transcriptional vector to generate an HIV-1 gag RNA template, which was further quantified and used as an HIV-1 RNA standard throughout this study. The other set of primers was designed by the proprietary software, LUX Desginer (Invitrogen Corp, http://www.invitrogen.com/lux), based on the HIV-1 gag RNA template generated by the first set of primers. All primers designed were finally checked using BLAST search (GenBank) and Primer Aligner Tools (Los Alamos HIV Database) to ensure the specific detection of all HIV-1 subtypes. To avoid any self-complementary and secondary structure (hairpin) formation, primers were checked using OligoAnalyser 3.0 program (http://207.32.43.70/biotools/oligocalc/oligocalc.asp). The details of the primers and the PCR product lengths are shown in Table 1. The first and second sets of primers were synthesized by Invitrogen Corp (USA) and PROLIGO (Singapore), respectively.

Production and quantitation of an RNA standard

To produce an RNA standard, the *gag* region of the HIV-1 genome was amplified by RT-PCR using specific primers (OutF and OutR). The one-step RT-PCR was performed according to the manufacturer's instructions using Qiagen® One-Step RT-PCR (QIAGEN, USA). The mastermix contained 50 μl of HIV-1 viral RNA templates, 400 μM for each: dNTP, 0.25 μM for each primer (OutF and OutR), 1X QIAGEN OneStep RT-PCR Buffer (include, 12.5 mM $MgCl_2$), 4 μl of QIAGEN OneStep RT-PCR Enzyme Mix (Omniscript, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase) and RNase-free water for a total volume of 100 μl. Amplification was performed in a Thermal Cycler (PCR System 9700; Applied Biosystems, Roche Molecular System, USA) as follows: 30 minutes at 50ºC for reverse transcription, 15 minutes at 95ºC for enzyme activation, 45 cycles of amplification (30 seconds at 94ºC, 30 seconds at 55ºC, 30 seconds at 72ºC), and 7 minutes at 72ºC for final extension.

After an RT-PCR step, each band of the PCR product was cut and extracted out of the gel using Qiagen® MinElute™ Gel Extraction kit (QIAGEN, USA) according to the manufacturer's instructions. Then, they were cloned in a pGEM®-T Easy Vector Systems (Promega, USA) as described by Sambrook *et al* (1989). The recombinant plasmids from the clones, which had an inserted *gag* region, were selected for verification by DNA sequencing at the BioService Unit or BSU (National Science and Technology Development Agency (NSTDA) Building, Bangkok, Thailand).

The recombinant plasmids were linearized by digestion with *Sal* I restriction endonuclease enzyme for 2-4 hours at 37ºC, purified by High Pure Viral Nucleic Acid kit (Roche, USA) accord-

Table 1
The primer sequences used for HIV-1 amplification and quantification.

Primer type	Primer name	Oligonucleotide sequence[a]	Product size(bp)
For *in vitro* RNA production			
Forward	OutF	5′ ATCACCTAGAACTTTGAATGCATG 3′	248 bp
Reverse	OutR	5′ CCCTCATCTGGCCTGGTGGA 3′	
For real-time RT-PCR			
Forward (FAM-labeled)	LUXF	5′ cagcttAGAAACCATCAATGAGGAAGCTG 3′	81 bp
Reverse	LUXR	5′ CTCATCTGGCCTGGTGGAAT 3′	

[a]The dye (FAM) was conjugated to the bold "T"; underlined bases denote a non-specific sequence built onto the primer to allow for hairpin conformation.

ing to the manufacturer's instructions, and then this served as a template for the production of an RNA molecule with T7 RNA polymerase. This reaction was performed according to the manufacturer's instructions using RiboMax™ Large Scale RNA Production System-T7 (Promega, USA) and carried out at 37ºC for 2-4 hours.

RNA produced by this *in vitro* transcription was treated with RNase-free DNase (Promega, USA) at a concentration of 1 U/μg of template DNA for 15 minutes at 37ºC, extracted with a phenol-chloroform-isoamylalcohol mix (25-24-1), chloroform-isoamylalcohol mix (24-1), and precipitated in ethanol (three volume) in the presence of 3 M sodium acetate pH 5.2 (1/10 volume) on ice for 2-5 minutes. The RNA pellet was carefully washed with 1 ml of 70% ethanol, then the pellet was dried at room temperature overnight. The RNA pellet was resuspended in nuclease-free water to a volume identical to that of the transcription reaction (100 μl) and quantified using the RNA-specific fluorescent dye RiboGreen® quantitaton kit (molecular Probes, USA) based on a LightCycler® instrument. The quantified standard RNA was then diluted to obtain 10^8 RNA copies/ml. Aliquots were stored at -80ºC in RNAse-free water.

Determination of HIV-1 RNA viral load by real-time RT-PCR using self-quenched fluorogenic primers

The real-time RT-PCR assay was performed in 20 μl PCR mixture volume consisted of 250 nM of each primer (FAM-labeled LUXF and Unlabeled LUXR), 250 ng/μl bovine serum albumin, and 1X SuperScript™ III RT / Platinum® *Taq* Mix (Invitrogen, USA) including 200 μM each dGTP, dATP, dCTP, and 400 μM dUTP, 3 mM $MgSO_4$, 20 mM, Tris-HCl (pH 8.4), 50 mM KCl, DNA polymerase antibodies, stabilizers, 1.2 U/ml Platinum® Taq DNA polymerase, and 5 μl RNA sample or 2 μl synthetic RNA (from 10^8 to 1 copies/ml), which acted as an external standard. The amplification was carried out as follows: reverse transcription at 45ºC for 30 minutes; initial activation of Platinum Taq DNA polymerase at 95ºC for 2 minutes; 65 cycles in three steps: 95ºC for 5 seconds, 60ºC for 10 seconds, and 72ºC for 4 seconds. At the end of the amplification cycles, melting temperature analysis was carried out by a slow increase in temperature (0.1ºC/second) up to 95ºC. Amplification, data acquisition and analysis was performed using a LightCycler® (Roche Molecular Biochemicals, Penzberg, Germany). The increase in the fluorescent signal was registered during the annealing step of the reaction.

For the intra-assay validation, three replicates were done for each scalar dilution, whereas for inter-assay analysis five experiments were performed. Synthetic RNA standard dilutions were employed as a reference curve when the clinical samples were assayed. HIV-1 RNA viral load final quantitative data were expressed as the number of copies per plasma milliliter, then transformed to log10 before being analyzed statistically.

Statistical analysis

The correlation and agreement relationship between real-time RT-PCR using LUX primers and Amplicor HIV-1 monitor kit were analyzed using two method comparison statistics: Deming regression and Bland and Altman plot.

RESULTS

Analytical sensitivity and specificity

The RNA standard quantified by the RiboGreen® RNA Quantitation reagent was used to generate an external standard curve for real-time RT-PCR. Fluorescence (Fig 1), measured at the end of each hybridization step, was plotted against the cycle number. The cycle number, at which fluorescence rose above the baseline level, was the C_T value. A regression curve was obtained for the C_T value versus the log concentration of a standard RNA plot. The HIV-infected individual plasma samples were amplified. Several dilutions of the RNA standard were tested (data not shown), from one million copies to one copy. When one copy of RNA was introduced in the PCR reaction, detection failed in most cases. Ten copies were always detected (confirmed by melting curve analysis).

The specificity of the RT-PCR reaction was tested with HIV-1 cultured in a 1A2 cell, the synthetic standard RNA, and the clinical samples from the HIV-1 infected patient plasma and the other RNA virus infected patient plasma (hepatitis C virus, influenza virus, and dengue virus

types 1-3). There was a small signal obtained from a non-specific sequence, but it had no detectable viral load (data not shown). The melting temperature was then used to confirm the specificity. The specific sequences (HIV-1 culture, synthetic RNA, clinical samples from HIV-1) had the same melting temperature (82ºC), whereas the non-specific sequences (other viruses) had a melting temperature of around of 77ºC, which was perhaps a primer dimer.

Accuracy and reproducibility

The reproducibility of the technique of both intra- and inter-assay using scalar dilutions of standard HIV-1 RNA was also tested. In particular, intra-assay reproducibility was evaluated using three replicates of each virus stock dilution between 10^6 and 10 HIV-1 RNA copies/ml (Fig 1). The coefficient variation (CV) for CT was <2.6% for all the standard scalar dilutions tested (Table 2). The CV for the copy number was always <16% for all the standard dilutions. Fig 1 shows the regression curve obtained for the evaluation of intra-run variation. Variability is more important for small numbers of copies in the PCR reaction. The inter-assay reproducibility was obtained employing five different experiments, indicating a CT CV <8.9% for all the standard scalar dilutions (Table 3). The CV of the copy numbers was always <25% for all the scalar standard dilutions.

Table 2
Comparison of the C_T for standard curves and intra-assay analysis.

Reference standard dilutions (copies/ml)	C_T mean values[a]	SD	%CV
10^6	29.48	0.21	0.72
10^5	32.90	0.25	0.76
10^4	36.54	0.61	1.68
10^3	36.63	0.44	1.19
10^2	40.42	0.65	1.62
10^1	41.56	1.06	2.54
10^0	ND	-	-

SD: Standard deviation; CV: Coefficient of variation; ND: not detected.
[a] For each sample, the C_T value is the average result from three replicates.

Table 3
Inter-assay analysis of C_T mean values.

Reference standard dilutions (copies/ml)	C_T mean values[a]	SD	%CV
10^8	16.93	0.68	4.03
10^7	21.08	0.94	4.47
10^6	25.02	1.06	4.23
10^5	28.01	1.19	4.25
10^4	32.93	1.98	6.01
10^3	37.16	3.28	8.83
10^2	40.75	1.28	3.14
10^1	42.73	1.77	4.15
10^0	ND	-	-

SD: Standard deviation; CV: Coefficient of variation; ND: not detected.
[a]For each sample, the C_T value is the average result from five different experiments (except 10^2 and 10^1, which were performed differently in three and two, respectively).

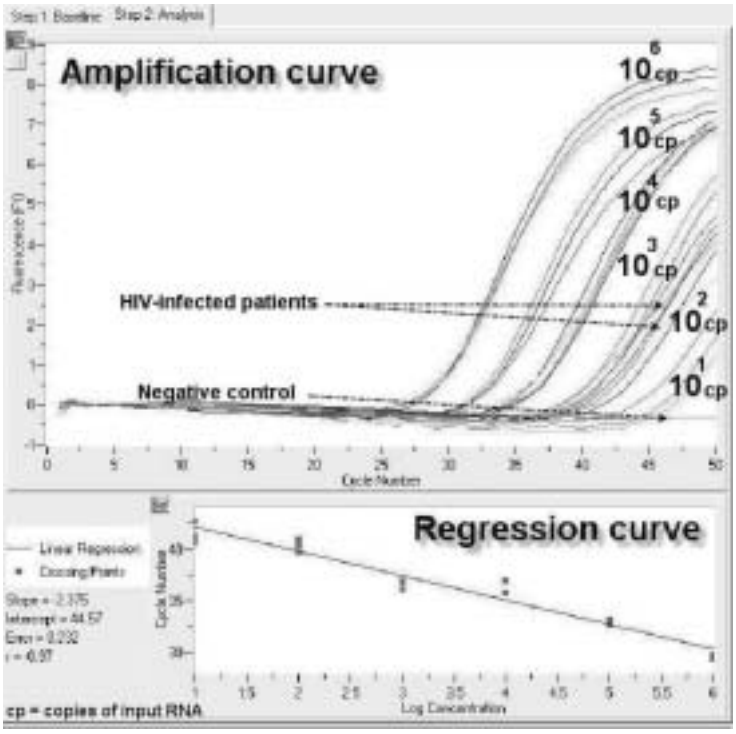

Fig 1–LightCycler RT–PCR of a serial dilution of seven standard RNA dilutions, repeated 3 times. Intra-run coefficient of variation (CV) was estimated with these three replicates for each dilution. The CV values are given in Table 2.

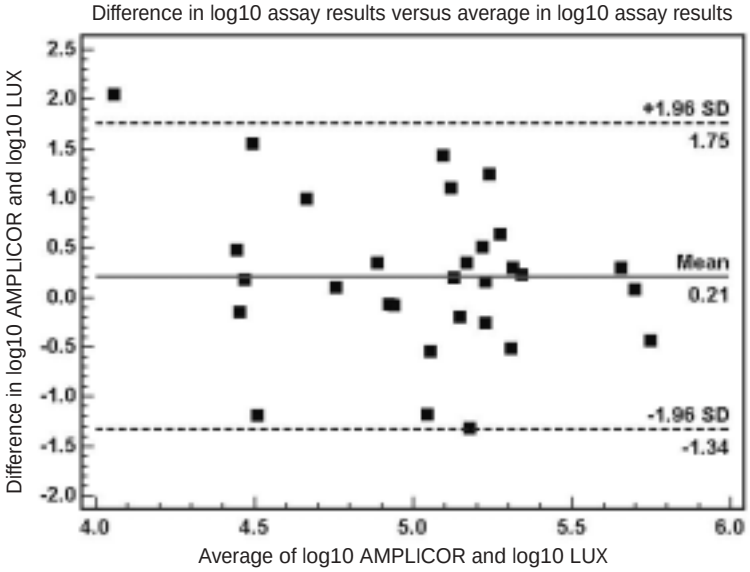

Fig 2–Difference in log10 for both assay results versus average for log10 of both assay results. The mean difference (solid line) ± 1.96 standard deviations (dashed lines) is shown. The plot shows that the different values are fairly homogeneously distributed between the 2 standard deviations (SD) above and below the mean (0.21).

Analysis and comparison of plasma viral load quantitation by real-time RT-PCR and AMPLICOR® HIV-1 Monitor assay in 50 cases of HIV-1patients

To test the self-quenched fluorogenic primer-based quantitative real-time RT-PCR assay on patient samples, viral RNA was extracted from 140 μl of plasma taken from 50 HIV-1 positive patients to measure HIV-1 viral load. As a reference curve, scalar dilutions (10^6 to 10 RNA copies/ml) of *in vitro* HIV-1 RNA were employed. The results (Table 4) demonstrate the real-time RT-PCR quantitative assay quantified 30 HIV-1 positive patient plasma samples, whereas 20 samples showed undetectable values confirmed by melting curve analysis (at 82ºC).

The AMPLICOR® HIV-1 Monitor assay represented a reference technique for quantitative determination of the HIV-1 RNA viral load in plasma. To correlate this reference technique to real-time RT-PCR using the LUX primer assay, this study tested the same 50 selected samples in parallel using the AMPLICOR® HIV-1 Monitor assay, a commercial kit. The results indicate that the AMPLICOR® HIV-1 Monitor assay and real-time RT-PCR using the LUX primer assay were in good agreement; mean difference in log10 copies/ml ± 2 standard deviations = 0.21 ± 1.34 (Fig 2).

DISCUSSION

The HIV-1 viral load in plasma is a major marker for the prognosis and therapy monitor-

Table 4
Cross section data of the viral load for each HIV-1 positive patient transformed into common (log10) logarithms.

Patients	AMPLICOR®	Real-time RT-PCR
Q1	5.46	5.23
Q2	5.31	5.15
Q3	5.05	5.57
Q4	4.56	4.38
Q5	5.59	4.96
Q6	5.05	5.25
Q7	5.23	5.03
Q8	5.43	Not detected
Q9	5.38	Not detected
Q10	5.06	4.72
Q11	5.65	Not detected
Q12	4.89	4.96
Q13	4.52	5.84
Q14	5.34	5.00
Q15	5.74	5.66
Q16	5.67	4.57
Q17	4.38	4.53
Q18	5.80	5.51
Q19	4.81	4.71
Q20	5.27	3.72
Q21	5.47	4.97
Q22	4.90	4.98
Q23	5.16	4.17
Q24	5.46	5.17
Q25	5.86	4.62
Q26	4.68	4.21
Q27	5.81	4.38
Q28	5.10	5.36
Q29	5.53	5.97
Q30	5.08	3.04
Q31	4.14	Not detected
Q32	4.12	Not detected
Q33	4.15	Not detected
Q34	4.36	Not detected
Q35	4.40	Not detected
Q36	4.72	Not detected
Q37	4.55	Not detected
Q38	4.16	Not detected
Q39	4.10	Not detected
Q40	4.45	5.64
Q41	3.56	Not detected
Q42	3.91	5.11
Q43	4.73	Not detected
Q44	3.33	Not detected
Q45	3.28	Not detected
Q46	3.15	Not detected
Q47	3.12	Not detected
Q48	4.78	5.33
Q49	3.57	Not detected
Q50	3.35	Not detected

ing of HIV-1 infection patients (Coombs, 1994; Cao *et al*, 1995; Mellors *et al*, 1995; Coombs *et al*, 1996; O'Brien *et al*, 1996), since this parameter directly reflects viral replication status (Mellors *et al*, 1997; Cozzi *et al*, 1998; HIV Surrogate Marker Collaborative Group, 2000) and treatment failure caused by emerging resistance to specific antiretroviral compounds (Katzenstein *et al*, 1996; Bartlett *et al*, 1998; Marschner *et al*, 1998). For these reasons, studies on molecular procedures to determine viral load quantitatively have increased in recent years. Recently, the advent of real-time RT-PCR offers a diagnostic system able to quantify specific amplicon synthesis during PCR cycles (Gibellini *et al*, 2004a,b; Ruelle *et al*, 2004).

In this paper, real-time RT-PCR evaluated by LightCycler® technology to measure HIV-1 viral load in patient plasma was developed. This method has ruled out the need for further manipulation of PCR products. This technique reduced the risks of contamination and allowing for the concomitant analysis of a large number of samples.

Lux™ technology was chosen for the detection of amplicons during the RT-PCR reaction. It provided a sensitivity and specificity that were comparable to dual-labeled probes and significantly greater than DNA binding dye. Moreover, it was compatible with a wide variety of real-time PCR instruments (Nazarenko *et al*, 2002a,b; Bedecarrats *et al*, 2003; Lowe *et al*, 2003). The Lux™ primers were designed using LUX Designer software from the sequencing result of the Outgag plasmid, which was constructed from the RT-PCR amplicons of the patient' s HIV infected plasma. *Gag* gene specific primers were selected because they are relatively well conserved and have been widely used in many previous studies (Vet *et al*, 1999; de Baar *et al*, 2001; Gibellini *et al*, 2004 a). From the alignment results using the PrimAlign tool (data not shown), these primers had a good alignment with many HIV-1 subtypes, especially subtypes E and B, being the major strains in Thai AIDS' patients (Ruxrungtham and Phanuphak, 2001).

The quantitation was based on an external standard curve obtained by 10-fold dilutions of the synthetic RNA produced by *in vitro* transcrip-

Table 5
Comparison of HIV viral load between the extraction kit and the initial input plasma volume in the Amplicor™ HIV-1 Monitor assay and the real-time RT-PCR using the LUX™ primer assay.

HIV viral load (copies/ml)				
Amplicor[a]	Qiagen[b]	Boom technique[b]		
plasma = 200 μl	plasma = 140 μl	plasma = 200 μl	plasma = 400 μl	plasma = 1000 μl
10^5	1,400	2,000	4,000	10^4
10^4	140	200	400	10^3
10^3	14	20	40	10^2
10^2	1.4	2	4	10^1
10^1	0.14	0.2	0.4	1
400[c]	5.6	8	16	40

[a]The final HIV copy number using an Amplicor™ HIV-1 Monitor assay (initial input plasma volume = 200 μl and input RNA template volume = 50 μl)

[b]The calculated HIV copy number when extracted by the Qiagen kit and Boom technique. HIV copies were applied to real-time RT-PCR by the LUX™ primer assay using 5 μl of RNA template in a final real-time reaction volume (20 μl).

[c]The lower limit of detection of the standard protocol of the Amplicor™ HIV-1 Monitor assay.

tion (T7 RNA polymerase). With standard synthetic RNA, an error can occur through spectrometry at the time of quantitation (Ruelle *et al*, 2004). To avoid that error, the RNA-specific fluorescent dye RiboGreen (Jones *et al*, 1998) which has widely been used in much research carried out on *in vitro* transcription technology (Cheung *et al*, 2004; Kuhlman *et al*, 2004), was used to measure the initial synthetic RNA concentration in this study.

The analytical sensitivity of this study demonstrated a large dynamic range of seven orders (10 to 10^6 copies/ml), with a strong linear correlation between the log10 of the initial copies and the fluorescence threshold cycle in accordance with other real-time techniques from previous publications (Niesters, 2001; Gibellini *et al*, 2004a,b; Ruelle *et al*, 2004).

Variability observed between or within runs was very low. The coefficient of variation calculated on C_T values remained below 1%. An eight percent coefficient of variability was reached for low copy numbers, which is acceptable and has no impact on clinical interpretation (Ruelle *et al*, 2004).

A direct clinical field evaluation of LUX™ primers RT-PCR was carried out by testing a small group of 50 HIV-infected patient plasma samples. In this cross-sectional analysis, the assay detected the viral load in 30 samples out of 50 (60%). When compared with the Amplicor™ HIV-1 Monitor assay (100%), the differences in performance between the two assays could be explained by initial input plasma volume and input RNA template volume (Table 5).

Table 5 demonstrates and answers why this study had a large range in sensitivity analysis. Some samples were not detected due to a viral load lower than 100,000 copies/ml. Viral loads (Amplicor assay) were 100,000 copies/ml for the patient's plasma, but when using the same sample in the developed assay, the RNA templates were only 1,400 copies/ml. To prove this hypothesis, the Boom technique was used to extract 6 plasma samples for each individual. The QIAamp® Viral RNA Mini Kit was limited to the initial plasma volume. The results (data not shown) supported the hypothesis: there was a trend of detecting low copy numbers using large plasma samples (1,000 μl) in accordance with other HIV viral loads based on real-time PCR and RT-PCR (Gibellini *et al*, 2004 a; Ruelle *et al*, 2004).

The presence of significant amounts of RNA secondary structure is also a parameter that influences the efficiency of the RT-PCR reaction.

Higher RT reaction temperatures can potentially alleviate RT pausing during cDNA synthesis by helping to melt RNA secondary structures (Wu *et al*, 1996; Harrison *et al*, 1998). They can then improve the reduction of amplification efficiency 4- to 10-fold as a target (Christopherson *et al*, 1997). Moreover, differences in the performance between assays that may affect these results may be due to multiple factors, including assay and biological variation, as well as specimen handling conditions (Alaeus *et al*, 1997; Ginocchio *et al*, 1997; Oelrichs *et al*, 1999). The linear regression and correlation were not performed in this real-time RT-PCR method and the Amplicor™ HIV-1 Monitor assay because those cross-sections of HIV viral load were not of normal distribution. However, there was good agreement (mean difference in log10 copies / ml ± 2 standard deviations = 0.21 ± 1.34) between the two methods (Fig 2). Bland and Altman (1986) pointed out that when a comparison of two clinical measurements of the same criterion is made, the use of the correlation may be misleading. The correlation coefficient measures the strength of the relationship between the variables, but does not necessarily measure the agreement between them (Bland *et al*, 1995; Highbarger *et al*, 1999; Dewitte *et al*, 2002).

In summary, this in-house HIV-1 quantification assay using self-quenched fluorogenic primers (LUX™ primers) based on real-time RT-PCR technology had a wide range linear relationship in analytical sensitivity and good reproducibility when tested with the scalar dilution of a synthetic RNA standard. They had good agreement with the reference assay, Amplicor™ HIV-1 Monitor assay, in high viral load samples. Although they could not detect low viral load samples, they were more sensitive at higher plasma volumes. Therefore, this technique might be used as a prototype for other PCR and RT-PCR real-time quantitative studies.

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