Establishment of Cheap and Reliable Real-Time PCR for Quantitation of HIV-1 Viral Load in Plasma

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Objective: *To establish an inexpensive and reliable real-time PCR for quantitation of HIV-1 RNA from plasma samples.*
Material and Method: *Previously analyzed 145 HIV-1 positive plasma samples with viral load ranging from less than 40 to approximately 1,000,000 copies/ml were included in the present study. HIV-1 gag gene was amplified and cloned into TA cloning vector. External standard curve was plotted using in vitro transcribed HIV-1 RNA and utilized for viral quantitation in the samples. Scramble nucleotides located in HIV-1 specific probe was subsequently constructed and used for individual systemic control. The correlation coefficient and Bland-Altman plot were applied for statistical analysis of the two methods.*
Results: *The limit of quantitation of the validated assay was 31 copies/ml and the linear range was approximate 31-1 x 10^7 copies/ml. After reproducibility determination using intra- and inter-run assay, it was implied that the coefficient of variation (%CV) was significantly increased while the low copy number of RNA was examined. A highly correlation (r^2 = 0.8099) and good agreement were obtained when the two assays were compared.*
Conclusion: *Developed real-time PCR was inexpensive and reliable for quantitation of HIV-1 viral load in plasma.*

Keywords: *HIV-1 RNA, viral load, real-time PCR*

J Med Assoc Thai 2012; 95 (12): 1563-8
Full text. e-Journal: *http://jmat.mat.or.th*

Human immunodeficiency virus type 1 (HIV-1) infection became an important issue of Acquired immunodeficiency syndrome (AIDS) worldwide. Thailand is one of AIDS epidemic area with approximately 370,000 people living with HIV-1[1]. Although there are effective programs to fight HIV-1 that are supported by the Thai government, there have been about 20,000 new infections every year. To improve the quality of life, infected individuals are currently encouraged by the national health insurance to undergo monitoring tests of at least two CD4+ cell counts, two plasma viral load tests, and one HIV-1 drug resistant test every year. HIV-1 viral load assay is used as a standard method for determination of the disease progression and the responsive against the anti-viral therapy[2]. Several HIV-1 monitoring test kits have been imported in Thailand. Although those tests are sensitive and easy, they are expensive. The cost per test of these commercial kits varies from 2,000 to 4,000 Baht (THB)[3]. Based on the highly sensitive, specific, and reduced carry-over contamination, real-time PCR have been widely used for detection and quantitation of various kinds of microorganisms including HIV-1[2,4,5].

In the present study, the authors attempted to find an inexpensive and reliable real-time PCR for quantitation of HIV-1 RNA load from plasma samples. In addition, scrambled sequence located in HIV-1 specific probe was constructed by splice-overlapped extension PCR (SOE-PCR) and its RNA was used as internal system control (IC) for detection of an ineffective PCR. HIV-1 *gag* RNA was *in vitro* transcribed and 10-fold serial diluted for standard curve generation utilized by Avogadro's equation. Viral RNA extracted from anonymous plasma samples was analyzed by validated real-time PCR and compared with the previously known copy number using reference test kit.

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Material and Method

Plasma samples

One hundred forty five left plasma samples were collected from routine viral load testing attending in Clinical Microbiology Service Unit (CMSU),

Faculty of Associated Medical Sciences, Chiang Mai University, between February and September 2011. Slightly hemolyzed plasma was also accepted in the present study[6]. These samples were previously examined using reference Cobas Ampliprep/Cobas Taqman HIV-1 test kit (Roche Diagnostics GmbH, Germany) and virus copy number was ranged from less than 40 to approximately 1,000,000 copies/ml. Moreover, twenty HIV-1 antigen and antibody negative plasmas were included for assay specificity determination.

HIV-1 gag *RNA and internal system control RNA synthesis*

Proviral DNA was extracted from HIV-1 positive dried whole blood and used as template for HIV-1 *gag* gene amplification using HIV-1 specific primers, HF and RH-gag (Table 1). The reaction mixture in 50 µl containing 5 µl 10X PCR buffer with $MgCl_2$ (iNtRON Biotechnology, Korea), 0.2 mM dNTPs, 0.4 µM of each primers, 2.5 U of *Taq* polymerase and 5 µl of extracted DNA. PCR profiling was performed in 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds with final extension at 72°C for 5 minutes. Amplified product (1,152 bp) was purified (Nucleospin extract II, Macherey-Nagel, Germany), ligated into pDrive TA cloning vector (QIAGEN® PCR Cloning kit, Qiagen, Canada) and transformed into *Escherichia coli* strain Top-10F.

To eliminate the false undetectable results caused by the PCR inhibitors, the artificial IC was constructed in the present study. Scramble sequence generated from the same nucleotide compositions as HIV-1 specific probe was amplified from proviral DNA by SOE-PCR using two pairs of primers, HF, ProR, and ProF, RH-gag (Table 1). The PCR reaction and profiling were conducted similar to as described above whereas the extension time was altered dependent on the size of amplified products (30 seconds and 50 seconds for a pair of HF, ProR and ProF and RH-gag, respectively). After amplification, full-length amplicon obtained from HF and RH-gag primers was gel purified, ligated into pGEM-T vector (pGEM-T easy vector system I, Promega, USA) and transformed into *E. coli*. The positive clones carried the target fragment were previously screened by colony-PCR using T7 and SP6 primers and the sequence integrity of extracted plasmid DNA were subsequently confirmed by automated DNA sequencing. *In vitro* transcription was performed according to the manufacturer's instruction (RiboMAX™ Large scale RNA production systems SP6 and T7, Promega, USA) for both HIV-1 *gag* RNA and IC RNA preparation. Briefly, plasmid DNA was linearized by *Sal* I restriction enzyme and used as template for RNA synthesis. Reaction was conducted in 100 µl containing 20 µl 5X T7 transcription buffer, 30 µl of rNTPs mixed, 10 µl of enzyme mix and 10 µg of lineared DNA. Reaction was gently mixed and incubated at 37°C for four hours. After that, the reaction tube was further treated with RNase free DNase enzyme to remove DNA template. Synthetic RNA was subsequently purified and reconstituted with RNase free water. To avoid ribonucleases contamination in long-term storage, 1 unit/µl RNase inhibitors (Toyobo Co. Ltd., Japan) was added and stored at -70°C until use.

Viral RNA isolation

Viral RNA was extracted from individual plasma according to manufacturer's instructions (High pure viral RNA kit, Roche Diagnostics GmbH, Germany). Prior to extraction, ten microliter corresponding to 1,000 copies of IC RNA was added into each plasma sample (200 µl). In addition, normal human plasma containing of IC RNA was used as a negative

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

Primer/probe	Sequence (5'-3')	Nucleotide position
HF	TACCCATGTTCTCAGCATTATC	1311-1332
HR	GATGGTTTCTTTTAACATTTGCA	1398-1420
RH-gag	GCCAAAGAGTGATTTGAG	2264-2281
Pro-F	CTACAATAGACTACACTAGCATAGCCTAAATATAGTGGG	-
Pro-R	TAGTGTAGTCTATTGTAGCTACCCTCTGATAATGCTGA	-
HIV probe	FAM-AGCCACCCCACAAGATTTAA ATATGATG-TAMRA*	1339-1366
IC probe	HEX-TAGCTACAATAGACTACACTAGCATAGC-TAMRA	1339-1366

* Fluorescent dyes tagged on probes are underlined

control in every run of in-house real-time PCR analysis.

Quantitation of HIV-1 RNA by Taqman based real-time PCR

Primers and TaqMan probe used in the present study were designed from the conserved *gag* region of HIV-1 subtype AE (Table 1). Real-time PCR reaction was undertaken in 20 µl containing of 10 µl of 2X master mix (RNA-direct™ Real time PCR Master Mix, Toyobo Co. Ltd., Japan), 2.5 mM Mn $(OAc)_2$, 0.3 µM of HF and HR primers, 0.25 µM of HIV-1 specific probe (5' FAM-3' TAMRA), 0.25 µM IC probe (5' HEX-3' TAMRA) and 5 µl of extracted RNA. The real-time thermal profiling was associated with two steps of reverse transcription and cycle amplification as followed: 90°C for 30 seconds and 61°C for 20 minutes and 48 cycles of 95°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds. Fluorescent signaling was analyzed by using real-time PCR machine (Chromo4™ Real-time PCR Detector, Bio-Rad Laboratories Incorporated, USA).

Standard curve construction and test limitation

Starting copy number of HIV-1 synthetic RNA was calculated following the Avogadro's equation[7-9]. HIV-1 synthetic RNA was ten-fold serially diluted in normal human plasma ranging from 10^8 to 10^2 copies/ml and analyzed by established real-time PCR. External master standard curve was generated in six replicate of each copy number against the cycle number where the signal raised over threshold cycle (C_T). The limit of quantitation was subsequently performed using two-fold serially diluted RNA from (15 to 1,000 copies/ml) and analyzed by real-time PCR.

Assay reproducibility

Assay reproducibility was evaluated by using intra- and inter-run assay. The percent of coefficient of variation (%CV) in at least six replications of the same and different runs were calculated (Table 2).

Statistical analysis

Bland-Altman plot[10] and Pearson's correlation coefficient (r^2) were applied for statistical analysis of our validated method and reference Cobas Ampliprep/ Cobas Taqman HIV-1 testing.

Results and Discussion

In the present study, an in-house real-time PCR was developed for quantitation of HIV-1 viral load from plasma and the calculated copy number was compared with the commercial HIV-1 monitoring test kit. Based on the highly conserved sequence, HIV-1 *gag* gene was selected for amplification by PCR. Approximately two-third of *gag* gene was amplified, ligated into TA cloning vector system, and transformed into *E. coli*. To eradicate the false negative results occurred by PCR inhibitors, internal system control was designed and constructed using SOE-PCR. Direct sequencing was carried out for sequence integrity analysis of both fragments and the chromatogram is shown in Fig. 1.

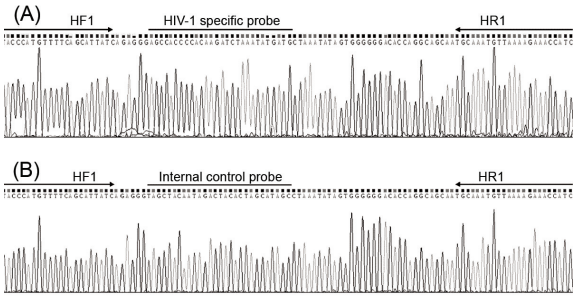

Fig. 1 The HIV-1 *gag* gene and internal control sequence was constructed and cloned into TA cloning vectors. The integrity of nucleotide compositions in both fragments was confirmed by direct sequencing and the chromatograms obtained from selected clones were illustrated. Primers (HF1, HR1) and probes regions of HIV-1 *gag* (A) and internal control sequence (B) were defined

In vitro transcription was generated for preparation of HIV-1 synthetic RNA and IC RNA. Avogadro's number was utilized for calculation of starting RNA copy number. Based on competitive PCR, copy number of IC RNA was initially optimized for

Table 2. The coefficient of variation (%CV) of intra- and inter-run assay

Calculated copy number	%CV
Intra-run (n = 6)	
3.0 E + 06	0.99
3.0 E + 05	1.88
3.0 E + 04	2.72
3.0 E + 03	2.18
3.0 E + 02	1.67
Inter-run (n = 8)	
3.0 E + 05	1.65
3.0 E + 04	2.26
3.0 E + 03	2.07
3.0 E + 02	4.58

interference determination of HIV-1 viral load. The result indicated that approximately 100 to 1,000 copies were appropriated for including into the validated real-time PCR system. The master standard curve was plotted between the various dilutions of HIV-1 synthetic RNA and the cycle number over the threshold and used for quantitation of viral load in clinical plasma. To assess the test limit of quantitation, the HIV-1 synthetic RNA was two-fold serially diluted in normal human plasma and analyzed by real-time PCR. The result indicated that the minimal detection was 31 copies/ml. The linear range of virus quantitation of established method was subsequently determined and the data showed an indifference of virus quantitation in both methods with the range of 31-1 x 10^7 copies/ml (the virus quantitation of reference commercial method ranged from 40-1 x 10^7 copies/ml). Test reproducibility was evaluated through the intra- and inter-run assay. The %CV was calculated and the results implied that %CV in each dilution was less variation ranging from 0.99 to 2.72 and 1.65 to 4.58 for intra- and inter-run assay, respectively. It was noticed that the %CV was significantly increasing while the low copy number of RNA was examined in different run. One hundred forty five HIV-1 positive plasmas with the viral load varied from less than 40 to 1,000,000 copies/ml were included in the present study. Among these samples, 35 of which were less than 40 copies/ml, 25 were 10^2 to 10^3 copies/ml, 25 were 10^3 to 10^4 copies/ml, 27 were 10^4 to 10^5 copies/ml, and 33 were 10^5 to 10^6 copies/ml. Twenty normal human plasmas previously screened with dual antigen and antibody testing, were applied for specificity determination. The results indicated none fluorescent signal detecting with HIV-1 specific probe was achieved in fifty-five samples of virus copy number < 40 copies/ml and normal plasmas. All of eighty-five samples (the viral load of 10^3 to 10^6 copies/ml) were accomplished using the validated test with over 76% (65/85) represented less than 1.0 log difference when comparing to the reference method. Unfortunately, approximately 32% (8/25) of 10^2 to 10^3 copies/ml were employed with the developed method. The plasma volume as well as long-term plasma storage might be related with undetectable results of HIV-1 low copy number[11,12]. An increasing of plasma volume has been previously reported to resolve this limitation[12-14]. Less than 1% (one sample with the plasma viral load of 431 copies/ml detected by the kit) was defined to be contaminated with PCR inhibitor thereby presenting no signal in dual fluorescent established method. Repeat was, therefore, unable in this sample due to the limitation of plasma volume. Albeit, PCR inhibition rarely appeared in the present study, up to 3.7% had been reported elsewhere[15]. Pearson's correlation coefficient (r^2) and Bland-Altman plot were used as statistical analysis for correlation study between the two methods. After the data analysis, the strength of linear association between the log values of validated method and Cobas Taqman reference method provided a highly correlated with calculated r^2 of 0.8099 and represented a good agreement between both methods (Fig. 2, 3). Linearity

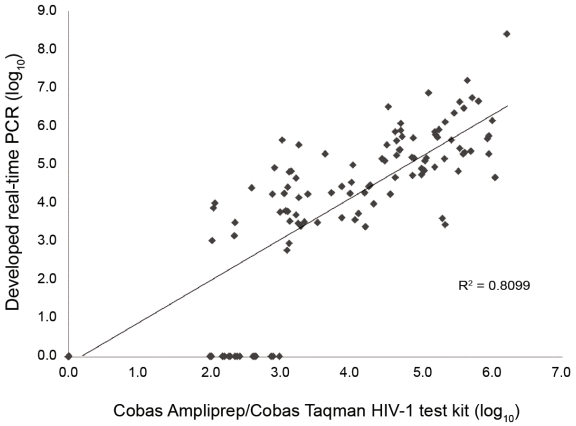

Fig. 2 The correlation analysis of developed real-time PCR assay (y-axis) and reference assay, Cobas Ampliprep/Cobas Taqman HIV-1 test kit (x-axis). The data represented highly correlate with correlation coefficient value (r^2) of 0.8099

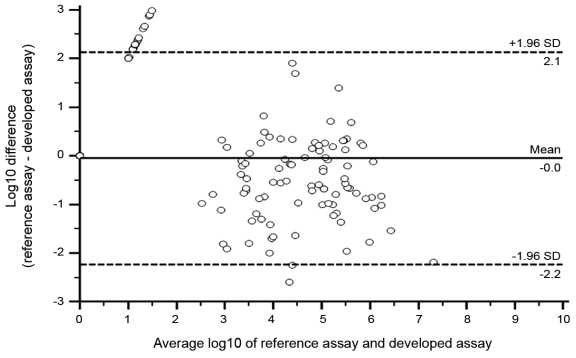

Fig. 3 An agreement analysis of the two techniques was measured by the Bland-Altman plot. The different of reference HIV-1 commercial kit and established real-time PCR (Y-axis) was plotted against the average in $\log_{10}$ of reference assay and validated assay (x-axis). The mean difference and ± 1.96 standard deviations (SD) are shown in solid line and dash lines, respectively. The open circle indicated each sample that was determined in this study

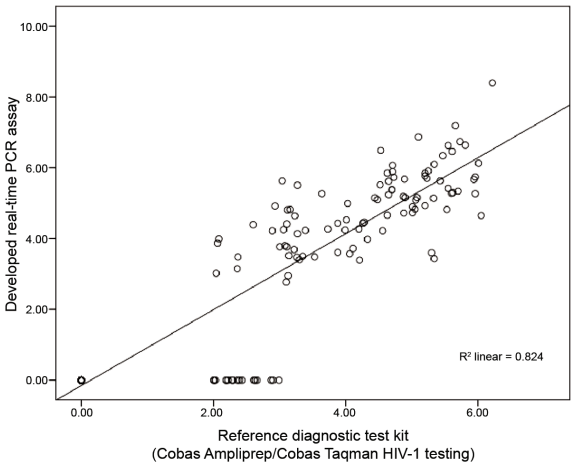

Fig. 4 The linearity study of the developed real-time PCR assay (y-axis) and reference HIV-1 commercial test kit (x-axis). The data indicated a good linearity between the two method ($r^2 = 0.824$)

study was analyzed, the data represented a good linearity between the two methods (Fig. 4). Moreover, the cost analysis of the validated method was compared to the commercial kit. Generally, cost of HIV-1 viral load testing was varied from 2,000 to 4,000 THB and depended on the company and the amount of purchasing. Except the cost of instrument and other hardware, the overall materials including consumed reagents were calculated and the result revealed approximately four times less expensive than the kit (560 vs. 2,000 THB). Therefore, the manual extraction protocol used in the present study might be limited while a number of plasma samples were assessed.

Conclusion

In summary, real-time PCR was generated for quantitation of HIV-1 RNA from plasma samples. HIV-1 *gag* RNA was *in vitro* transcribed and applied for external standard curve construction. Additionally, IC RNA was alternatively transcribed and used as individual systemic control for investigation of PCR inhibition. Intra- and inter-run assay were inquired for reproducibility determination of the validated method. The statistical analysis represented a highly correlation and exhibited a good agreement indicating an indifferent between the two methods. Developed real-time PCR was cost effective and reliable method for HIV-1 viral quantitation in plasma samples in resource-limited countries especially in Thailand.

Funding source

The present study was financial supported by Office of the Higher Education Commission, Thailand.

Acknowledgements

The authors want to thank Mr. Praphan Luangsuk, Head of Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University for helpful comments and Assoc. Prof. Luksana Makonkawkeyoon and Mr. Somphon Pharephan, Department of Biochemistry, Faculty of Medicine, Chiang Mai University for supporting equipment and some chemicals.

Potential conflicts of interest

None.

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การพัฒนาเทคนิค Real-time PCR ที่มีราคาถูกและน่าเชื่อถือเพื่อตรวจหาปริมาณไวรัส HIV-1 จากพลาสมา

คณิตาพร สุภาเดช, สรศักดิ์ อินทรสูต

วัตถุประสงค์: *เพื่อพัฒนาเทคนิค Real-time PCR ที่มีราคาถูกและน่าเชื่อถือสำหรับการตรวจหาปริมาณไวรัส HIV-1 จากตัวอย่างพลาสมา*

วัสดุและวิธีการ: *ในการศึกษาครั้งนี้ได้ใช้ตัวอย่างพลาสมาที่มีปริมาณเชื้อไวรัสตั้งแต่น้อยกว่า 40 ถึง 1,000,000 copies/ml จำนวน 145 ราย มาทำการตรวจวิเคราะห์ โดยทำการสร้างและสังเคราะห์อาร์เอ็นเอจากจีน gag ของเชื้อไวรัส HIV-1 ด้วยเทคนิค in vitro transcription จากนั้นนำไปสร้างกราฟมาตรฐานเพื่อใช้ในการคำนวณหาปริมาณไวรัสจากตัวอย่างพลาสมา นอกจากนี้ลำดับนิวคลีโอไทด์ในส่วนของโพรบที่จำเพาะกับ HIV-1 ได้ออกแบบให้สลับตำแหน่งเพื่อสร้างอาร์เอ็นเอสำหรับควบคุมระบบ ค่าสถิติที่ใช้ในการศึกษาความสัมพันธ์ของวิธีที่พัฒนาขึ้นกับวิธีการมาตรฐานในการศึกษาครั้งนี้ คือค่าสัมประสิทธิ์ความสัมพันธ์ และ Bland-Altman plot*

ผลการศึกษา: *วิธี Real-time PCR ที่พัฒนาขึ้นสามารถตรวจหาปริมาณอาร์เอ็นเอของเชื้อไวรัส HIV-1 ได้ต่ำที่สุดที่ 31 copies/ml และสามารถคำนวณหาปริมาณไวรัสได้ตั้งแต่ 31 ถึง 1 x 10^7 copies/ml จากการศึกษา reproducibility ของวิธีที่พัฒนาขึ้น พบว่าค่า %CV (Coefficient of variation) ที่ได้มีค่าสูงขึ้นเมื่อปริมาณของอาร์เอ็นเอมีค่าต่ำ เมื่อเปรียบเทียบวิธีที่พัฒนาขึ้นกับวิธีมาตรฐานพบว่ามีความสัมพันธ์สอดคล้องกันด้วยค่าสัมประสิทธิ์สหสัมพันธ์ เท่ากับ 0.8099*

สรุป: *เทคนิค Real-time PCR ที่พัฒนาขึ้น มีราคาถูก และมีความน่าเชื่อถือ สามารถนำไปใช้เพื่อตรวจหาปริมาณไวรัส HIV-1 จากตัวอย่างพลาสมาได้ต่อไป*