CHAPTER 4

RESULTS

1. HIV-1 *gag* gene and internal system control construction

Based on the highly conserved sequence, HIV-1 *gag* gene was selected for amplification by PCR. After proviral DNA extraction, it was used as template for HIV-1 *gag* gene amplification using HF1 and RH-*gag* primers. An approximately 956 bp or two-third of *gag* gene was amplified and amplicon was subsequently gel purified, ligated in to pDrive TA cloning vector system and transformed into *E.coli* (Figure 9). Colony-PCR technique was performed for selection of positive cloned that carried the HIV-1 *gag* gene. T7 and SP6 specific primers were used and the PCR amplified product of predicted to be 1,152 bp (Figure 10).

To determine the false undetectable results caused by PCR inhibitors, internal system control or IC was alternatively synthesized by using Spice overlapped extension-PCR (SOE-PCR). The sequence located in the HIV probe binding was scrambled and separately amplified from proviral DNA using two pairs of primers; HF, ProR and ProF, RH-*gag*. After SOE-PCR reaction, the predicted DNA product, approximately 1,043 bp, was purified, ligated in to pGEM-T Easy vector system and transformed into *E. coli* (Figure 11). The positive clones that carried an inserted gene were selected by using T7 and SP6 primers (1,213 bp). For integrity determination,

both positive clones that carried the target gene were propagated and purified. Direct sequencing was performed and the chromatogram was shown in figure 12.

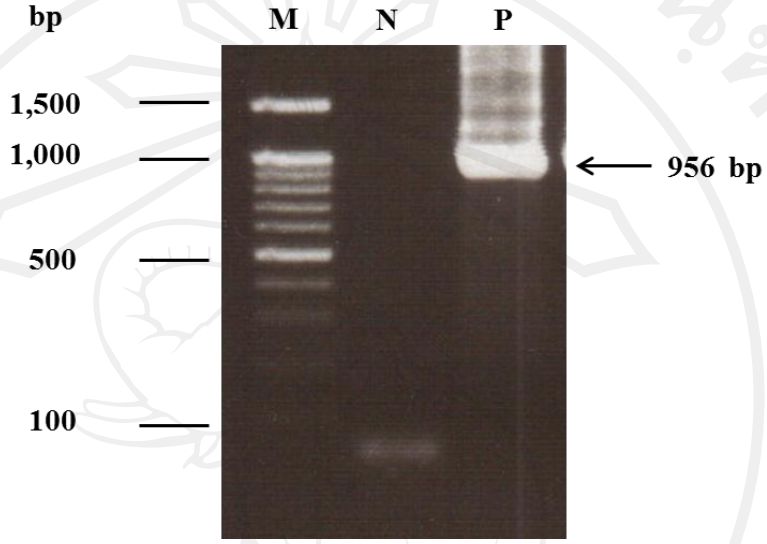

Figure 9 1.5 % agarose gel electrophoresis of HIV-1 *gag* gene amplification by PCR.

Lane M = 100 bp DNA marker

Lane N = Negative control

Lane P = An amplified product (956 bp).

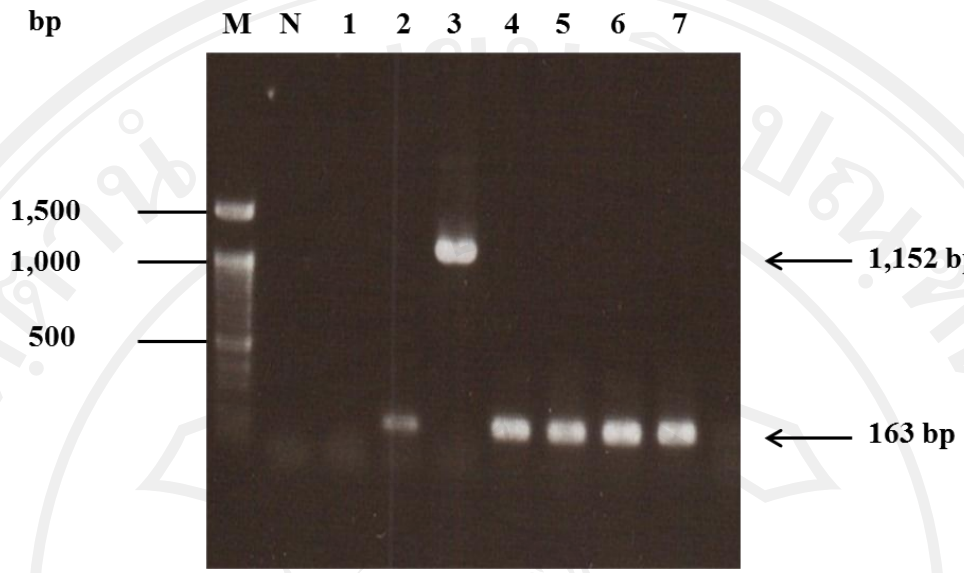

Figure 10 The colony-PCR amplification for detection of a positive clone.

The colony-PCR was used in this study for screening of the positive plasmid that carried the HIV-1 *gag* gene. Amplified product was electrophoresed in 1.5% agarose gel, stained with ethidium bromide and analyzed under UV transilluminator. The positive target gene inserted into the vector and no-insert gene was calculated to be approximately 1,152 bp and 163 bp, respectively.

Lane M = 100 bp marker

Lane N = Negative control

Lane 1, 2, 4-7 = PCR product amplified from no-insert plasmid.

Lane 3 = Amplified product from the positive target gene inserted into the vector

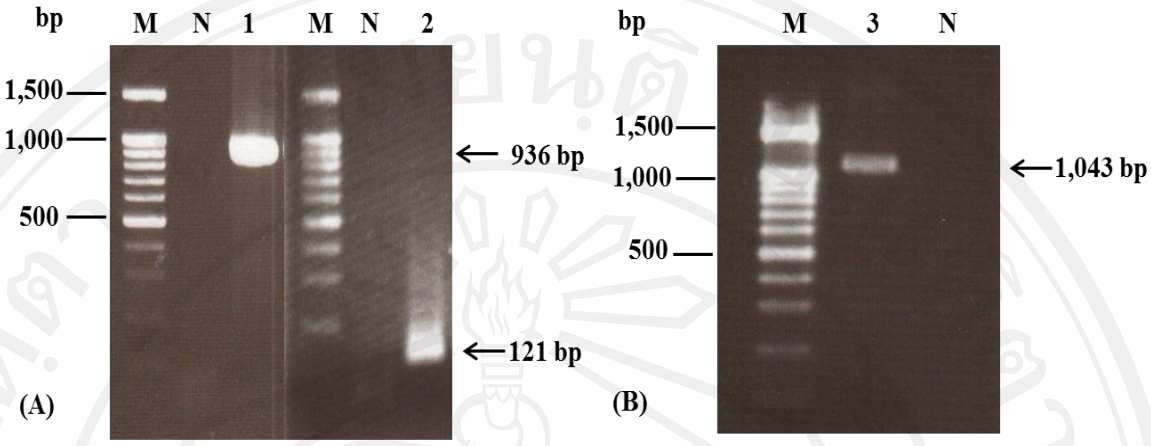

Figure 11 Internal system control construction by Splice overlapped extension-PCR (SOE-PCR).

Nucleotide sequence located in HIV-1 specific probe was mixed and used for primers synthesis. HIV-1 *gag* gene franking with probe region were separately amplified using two sets of primers: ProF-RH*gag* primers and pDF-ProR primers. The calculated amplified products were 936 and 121 bp, respectively (A). These two amplified fragments were mixed and re-amplified using pDF and RH-*gag* primers and the final product was approximately 1,043 bp (B).

Lane M = 100 bp standard marker

Lane N = Negative control

Lane 1 = PCR product amplified from ProF-RH*gag* primers (936 bp)

Lane 2 = PCR product amplified from ProF-RH*gag* primers (121 bp)

Lane 3 = PCR product from SOE-PCR (1,043 bp)

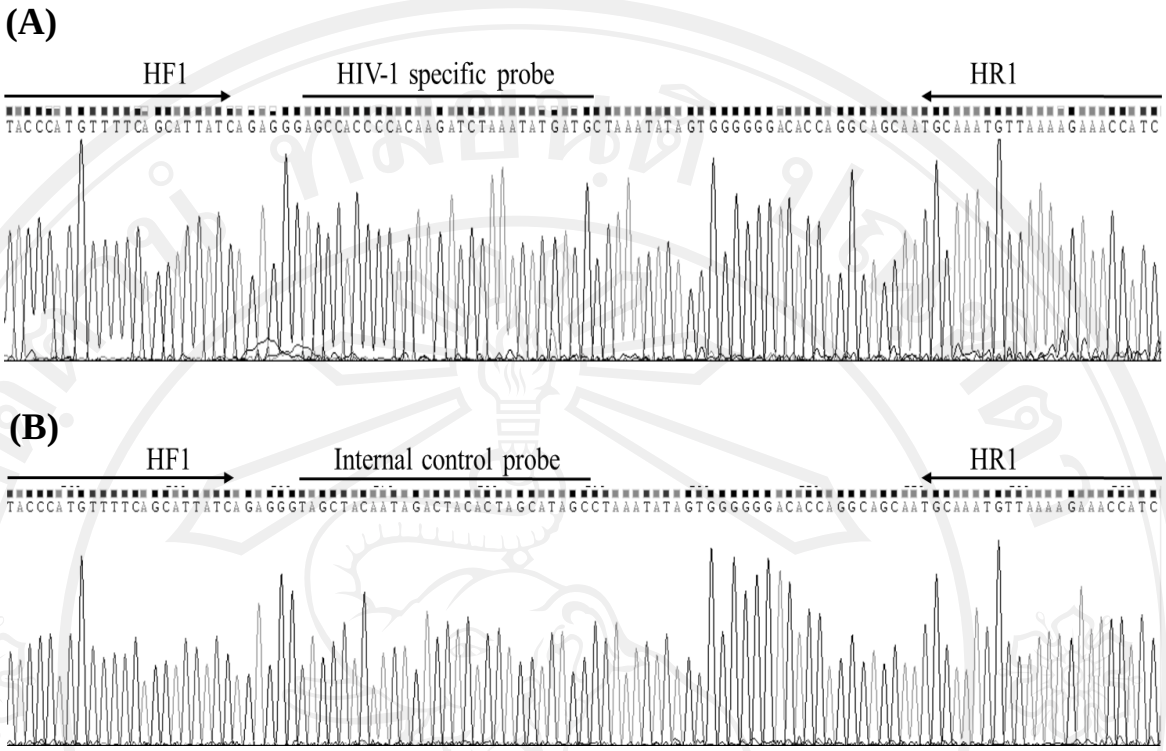

Figure 12 Chromatogram of direct sequencing for integrity determination of HIV-1 *gag* and internal system control sequence.

Sequence of HIV-1 specific probe (A) and internal system control probe (B) were located in line. Arrows demonstrated the location of HIV-1 specific primers, HF1and HR1.

2. *In vitro* transcription for HIV-1 *gag* RNA and internal system control synthesis

Plasmids carried HIV-1 *gag* gene and internal system control gene were purified and linearized by using *Sal*I restriction enzyme. The linear DNA was used as template for *in vitro* transcription. After DNase treatment, synthetic RNA was precipitated and analyzed by agarose gel electrophoresis (Figure 13).

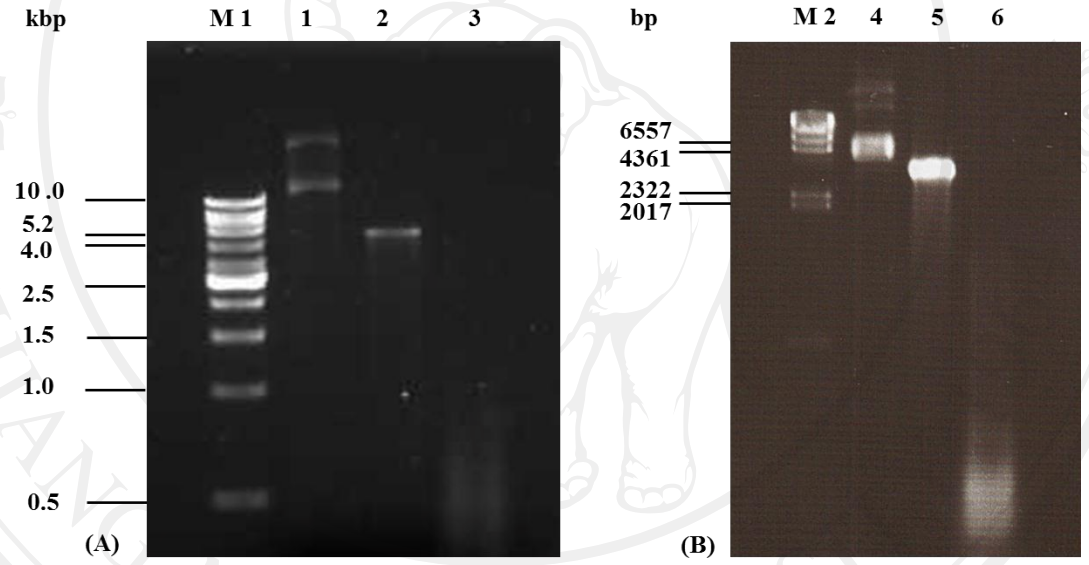

Figure 13 *In vitro* transcription for HIV-1 *gag* RNA and HIV-1 internal system control synthesis.

Lane M_1 and M_2 = 1 kb DNA marker and Lambda *Hind*III DNA marker

Lane 1-3 = Purified plasmid DNA, linearized plasmid DNA and HIV-1 *gag* RNA, respectively

Lane 4-6 = Purified plasmid DNA, linearized plasmid DNA and internal system control RNA, respectively.

3. Standard curve construction and test limitation

Avogadro's equation was utilized for copy number calculation of both synthetic HIV-1 *gag* RNA and internal system control RNA. The undiluted HIV-1 *gag* RNA was calculated to be approximately 10^{13} copies/ml. The synthetic RNA was subsequently diluted in 10-fold serially dilution in normal human plasma ranging from 10^{5}-10^{10} copies/ml and analyzed by using validated assay. For test validity, about 1,000 copies/ml of internal system control RNA were individually mixed into these plasma samples. Each dilution of synthetic RNA was determined in six replicates. Standard curve was plotted between the HIV-1 synthetic RNA dilution and the cycle number over the threshold and applied for virus quantitation in HIV-1 positive plasma samples (Figure 14). In this study, the optimal amplification cycle was 48 cycles. After the extrapolated line was constructed, the range of virus quantitation using validated method was approximately 10^{3}-10^{10} copies/ml.

4. Optimization of internal system control RNA

Internal system control RNA (IC-RNA) was optimized for minimal interference determination of validated real-time PCR. IC-RNA was 10-fold serially diluted from 10^{3}-10^{7} copies/ml with normal human plasma containing an approximately 10^{3}-10^{4} copies/ml of HIV-1 RNA standard. The selected copies number of IC-RNA used for validated assay was 10^{3} copies/ml (Figure 15-16).

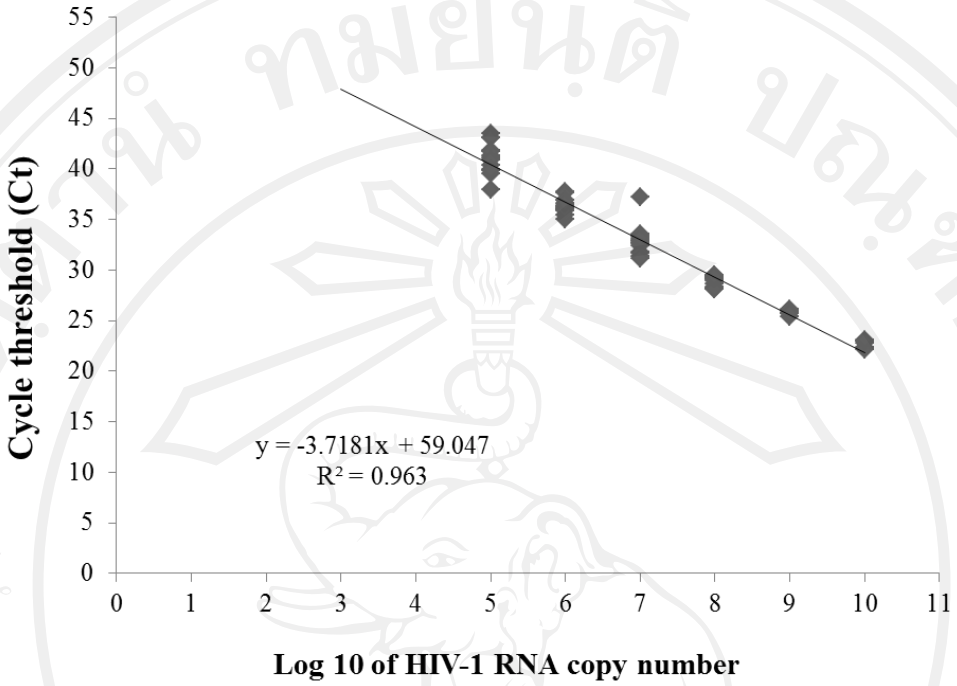

Figure 14 External standard curve for HIV-1 virus quantitation using validated assay.

The linear regression line was plotted between the threshold cycle (Ct) values (Y-axis) and log 10 of HIV-1 *gag* RNA copy number generated from synthetic RNA (X-axis). Each dilution was done in six replicates.

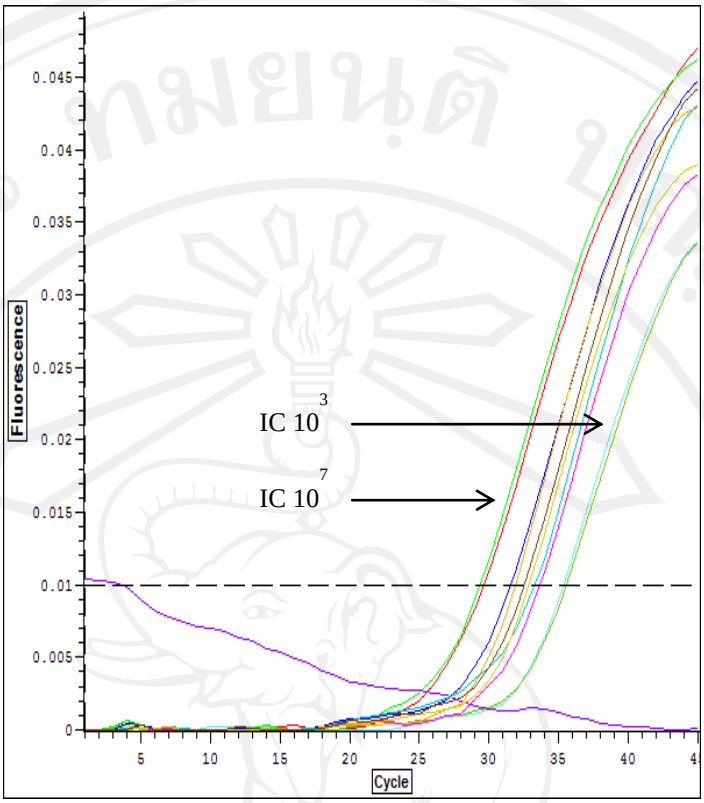

Figure 15 The cut-off determination of internal system control RNA (IC-RNA).

The fluorescent cut-off used for internal system control was optimized. IC-RNA was 10-fold serially diluted from 10^3-10^7 copies/ml in normal human plasma with the lacking of HIV-1 *gag* RNA. The appropriated fluorescent threshold cut-off was decided to be 0.01.

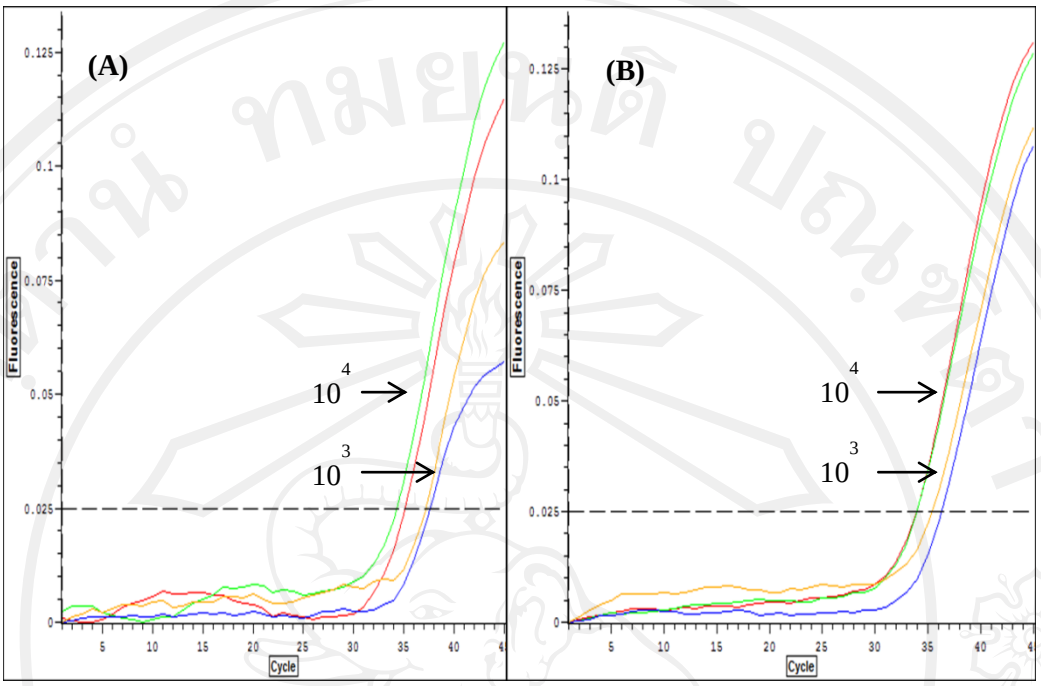

Figure 16 Interference determination of internal system control (IC-RNA) using validated method.

The copy number of internal system control was determined for assay interference. HIV-1 *gag* RNA was 10-fold serially diluted from 10^3-10^4 copies/ml in normal human plasma with the lacking of IC-RNA (A) and in the present of IC-RNA (10^3 copies) (B). The fluorescent threshold cut-off used for internal control and individual HIV-1 quantitation was previously analyzed and assigned to be 0.01 and 0.025, respectively.

5. Assay reproducibility

The reproducibility of the assay was evaluated through the intra- and inter-run assay. The intra-run assay was performed to test the variation within the same run and the inter-run assay was considered for the variation between different run. Regarding to intra-run assay, six replicates of serial ten-fold dilution of HIV-1 *gag* synthetic RNA ranging from 10^3-10^9 copies/ml were examined whereas 10^5-10^8 copies/ml of HIV-1 *gag* RNA were determined in eight different days for inter-run analysis. The percentage of coefficiency of variation (%CV) and mean of Ct values were calculated and represented in table 3 and 4.

Table 3 Reproducibility determination of intra-run assay (n=6) using the validated real-time PCR.

Copy number (copies/ml)	% CV	Ct Mean value
1E+03	5.41	42.78
1E+04	2.39	41.12
1E+05	1.81	40.93
1E+06	2.18	36.55
1E+07	2.72	32.31
1E+08	1.67	29.12
1E+09	0.99	25.81

Table 4 Reproducibility analysis of inter-run assay (n=8) using the validated real-time PCR.

Copy number (copies/ml)	% CV	Ct Mean value
1 E+05	4.58	40.92
1 E+06	2.07	36.21
1 E+07	2.26	32.60
1 E+08	1.65	28.76

6. Determination of HIV-1 positive plasma samples using validated real-time PCR

A total of 232 plasma samples including 40 sero-negative plasma samples and 192 HIV-1 positive samples, were determined in this study. These HIV-1 positive samples were previously examined using reference kit and viral load was varied from less than 40 to $1.7x10^6$ copies/ml. It was indicated as undetectable results in all sero-negative (Figure 17) and all HIV-1 positive plasma samples with viral load less than 40 copies/ml since fluorescent signal was unable to observe after HIV-1 probe labeled with FAM-TAMRA is present.

The results in log10 values of reference and validated real-time PCR and the log10 difference values were shown in separate ranges of viral load (Table 6-10). When comparing with the reference method, about fifty percent (93/192) of the viral load less than 1.0 log difference was observed in the validated assay. The percent and the number of samples with viral load less than 1.0 log difference of validated real-time PCR assay in comparison to reference test was shown in table 5. However, five samples (Approximately 2.6%) with the plasma viral load of 431, $1.66x10^5$, $2.5x10^5$, $3.75x10^5$ and $5.13x10^6$ copies/ml determined by reference commercial method, revealed no fluorescent signal in both IC and HIV-1 RNA detection by the validated assay. An example of the fluorescent signaling curves of the positive plasma samples tested by the validated assay was represented in figure 18.

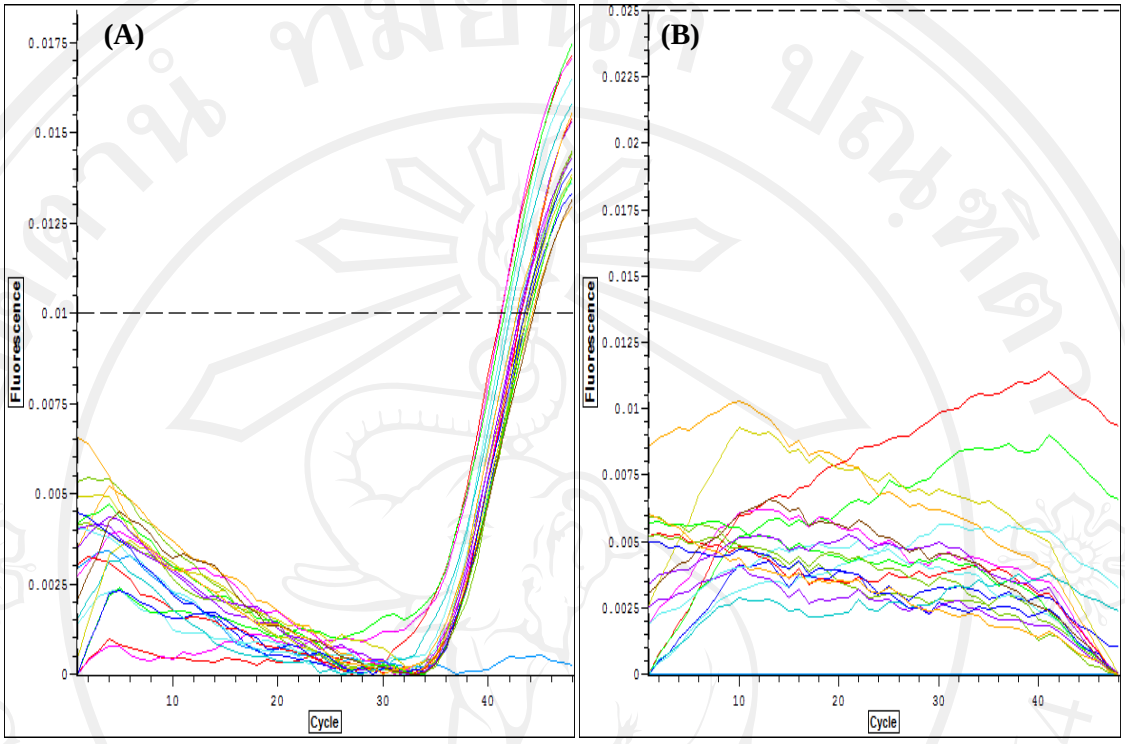

Figure 17 The illustration of the fluorescent signaling curve obtained from sero-negative plasma samples after tested with validated real-time PCR assay.

Fluorescent signaling curve obtained from HEX-TAMRA probe (A) and FAM-TAMRA probe (B) was analyzed. Y- and X-axis was fluorescent intensity and cycle number, respectively. The fluorescent threshold cut-off used for internal control and for individual HIV-1 quantitation was 0.01 and 0.025, respectively.

Table 5 Determination of HIV-1 positive plasma samples using validated real-time PCR and comparison with reference method.

Copy number (copies/ml)	n	The comparison of validated assay and reference kit	
		Less than 1.0 log different	Less than 1.0 log different (%)
< 40	19	19	100.00
40 - 10^2	31	0	0.00
10^2-10^3	40	2	5.00
10^3-10^4	31	19	61.29
10^4-10^5	30	24	80.00
10^5-10^6	41	29	70.73
Total	192	93	48.44

Table 6 The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (40-100 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference(Reference-validated)
1.49	0.00	1.49
1.61	0.00	1.61
1.66	0.00	1.66
1.71	4.96	-3.25
1.72	0.00	1.72
1.75	0.00	1.75
1.79	0.00	1.79
1.80	0.00	1.80
1.83	4.91	-3.09
1.85	0.00	1.85
1.85	0.00	1.85
1.85	4.85	-2.99
1.87	0.00	1.87
1.89	0.00	1.89
1.89	6.10	-4.21

Table 6 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (40-100 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
1.90	0.00	1.90
1.90	4.87	-2.97
1.91	0.00	1.91
1.91	4.19	-2.28
1.91	5.10	-3.18
1.92	4.50	-2.57
1.93	7.08	-5.15
1.93	0.00	1.93
1.95	0.00	1.95
1.96	0.00	1.96
1.96	0.00	1.96
1.96	0.00	1.96
1.96	0.00	1.96
1.99	4.33	-2.34
1.99	0.00	1.99
2.00	5.35	-3.36

Table 7 The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (100-1,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
2.03	0	2.03
2.04	0	2.04
2.04	3.02	-0.98
2.05	0	2.05
2.06	3.87	-1.81
2.06	0	2.06
2.08	3.99	-1.91
2.13	0	2.13
2.14	0	2.14
2.15	0	2.15
2.16	0	2.16
2.18	0	2.18
2.22	0	2.22
2.23	0	2.23
2.31	0	2.31
2.33	0	2.33

Table 7 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (100-1,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
2.36	0	2.36
2.36	3.15	-0.80
2.37	3.48	-1.11
2.51	4.67	-2.16
2.55	0	2.55
2.56	0	2.56
2.59	0	2.59
2.60	4.39	-1.79
2.61	0	2.61
2.62	4.79	-2.16
2.63	0	2.63
2.69	0	2.69
2.70	0	2.70
2.73	5.03	-2.31
2.81	4.17	-1.35
2.83	0	2.83

Table 7 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (100-1,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
2.87	0	2.87
2.89	4.22	-1.33
2.89	0	2.89
2.93	4.92	-1.99
2.94	0	2.94
2.94	0	2.94
2.96	5.21	-2.24
2.96	0	2.96

Table 8 The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (1,000-10,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
3.00	3.77	-0.77
3.02	4.51	-1.49
3.04	5.63	-2.60
3.06	4.25	-1.19
3.08	3.80	-0.71
3.10	2.77	0.33
3.11	3.77	0.66
3.11	4.41	-1.30
3.12	4.81	-1.69
3.13	2.95	0.18
3.14	3.52	0.38
3.16	4.82	1.66
3.22	3.69	-0.47
3.22	3.15	0.07
3.23	4.64	1.41
3.26	3.47	-0.21

Table 8 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (1,000-10,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
3.27	5.51	-2.25
3.27	4.14	-0.87
3.27	4.78	-1.51
3.30	3.40	-0.10
3.30	4.48	-1.18
3.35	3.50	-0.16
3.39	4.23	-0.84
3.39	4.35	-0.95
3.53	3.48	0.05
3.57	5.06	-1.49
3.64	5.27	-1.63
3.73	4.27	0.54
3.88	4.43	0.55
3.88	3.61	0.27
3.99	4.24	0.25

Table 9 The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (10,000-100,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
4.01	4.53	-0.52
4.03	4.99	0.96
4.06	3.57	0.49
4.11	3.72	0.39
4.20	4.27	0.07
4.21	3.39	0.82
4.26	4.43	-0.17
4.28	4.46	-0.17
4.33	3.98	0.35
4.44	5.15	-0.71
4.48	5.10	-0.62
4.52	5.52	-1.00
4.53	6.49	-1.96
4.56	5.71	-1.16
4.56	4.22	0.34
4.60	6.31	-1.71

Table 9 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (10,000-100,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
4.63	4.66	-0.03
4.63	5.85	-1.22
4.65	5.24	-0.59
4.65	5.62	0.97
4.70	5.38	-0.68
4.70	5.38	-0.69
4.71	5.89	-1.18
4.71	6.07	-1.37
4.73	5.73	-1.00
4.85	4.48	0.37
4.87	5.19	0.32
4.88	4.72	0.16
4.89	5.68	-0.79
4.90	5.16	-0.26

Table 10 The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (100,000-1,700,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
5.01	4.73	0.28
5.01	4.90	0.11
5.05	4.83	0.22
5.06	5.09	0.03
5.08	5.16	0.08
5.10	6.87	-1.76
5.20	4.93	0.27
5.20	5.85	-0.65
5.20	5.77	-0.57
5.23	5.70	0.47
5.24	5.07	0.17
5.24	4.98	0.26
5.25	5.91	-0.66
5.30	3.60	1.70
5.33	5.14	0.19
5.33	6.69	-1.36

Table 10 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (100,000-1,700,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
5.34	6.10	-0.76
5.34	3.43	1.91
5.43	5.63	-0.20
5.47	6.34	0.87
5.53	4.82	0.71
5.54	6.57	-1.03
5.55	5.42	0.13
5.55	6.63	-1.08
5.58	5.79	-0.21
5.60	5.28	0.32
5.61	6.46	-0.85
5.62	5.30	0.32
5.66	7.19	-1.54
5.70	5.34	0.36
5.70	5.84	-0.14
5.73	6.74	-1.01

Table 10 (continued) The log10 values of HIV-1 positive plasma samples determined by the validated and reference real-time PCR assay and the log10 difference between both methods (100,000-1,700,000 copies/ml).

(CobasAmpliPrep / CobasTaqman HIV-1 test (log10)	Validated assay (log10)	Log10 difference (Reference-validated)
5.81	6.64	0.83
5.94	5.67	0.27
5.96	5.27	0.68
5.96	5.74	0.21
6.01	6.13	-0.13
6.05	4.65	1.40
6.14	3.55	2.59
6.22	8.40	-2.18
6.24	7.32	-1.08

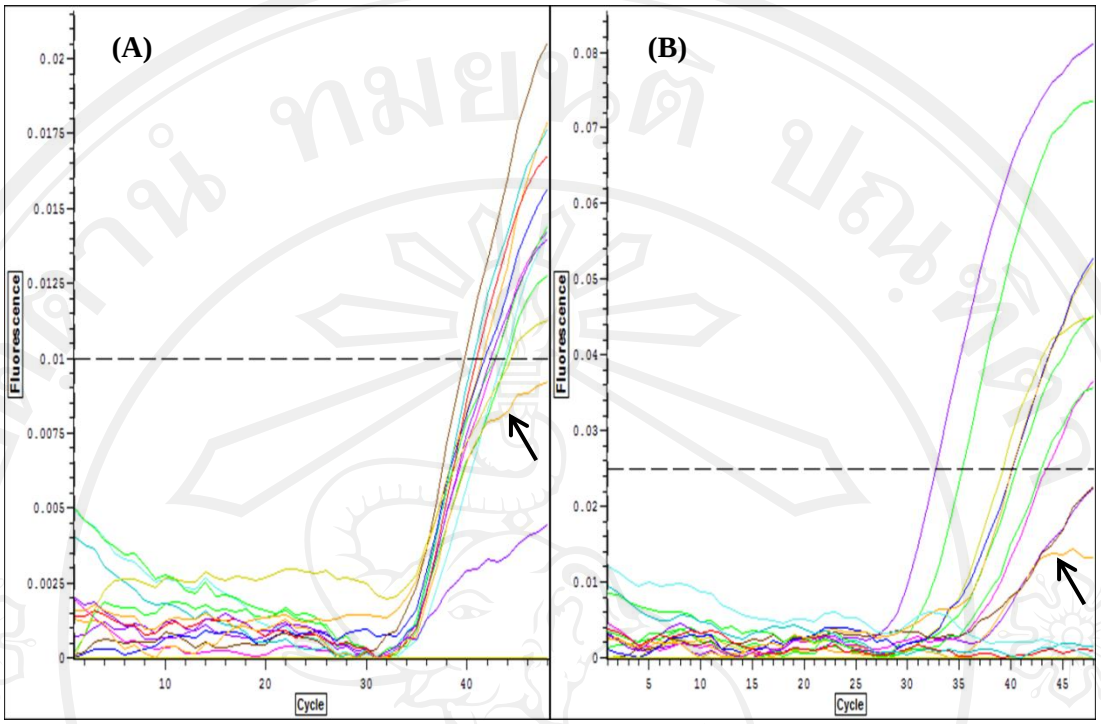

Figure 18 The demonstration of fluorescent signal obtained from 15 positive plasma samples after examined with validated real-time PCR assay.

Fifteen positive plasma samples were determined by our established real-time PCR. Fluorescent signal obtained from HEX-TAMRA probe (A) and FAM-TAMRA probe (B) was depicted. Y- and X-axis was fluorescent intensity and cycle number, respectively. One positive plasma sample that virus copy number was previously analyzed to be 431 copies/ml, revealed no fluorescent signal in both IC-RNA and HIV-1 virus detection (Arrows).

7. The correlation and agreement analysis of the validated assay

Correlation coefficient and Bland-Altman plot were used for statistical analysis of the two methods. The linear regression relationship between the log10 values of validated assay and reference method was plotted and the correlation coefficient (r^2) of all plasma samples analyzed was calculated to be 0.6843 (Figure 19). Therefore, the correlation analysis using plasma viral load ranging from 10^3–$1.7x10^6$ copies/ml revealed a good correlation with r^2 value of 0.9032 (Figure 20). An agreement analysis of both methods was plotted by using the log10 difference between both methods (Y-axis) against the average in log10 of reference kit and validated assay (X-axis). The plot demonstrated that the different values were homogeneously distributed within 2 standard deviations (SD) (Figure 21).

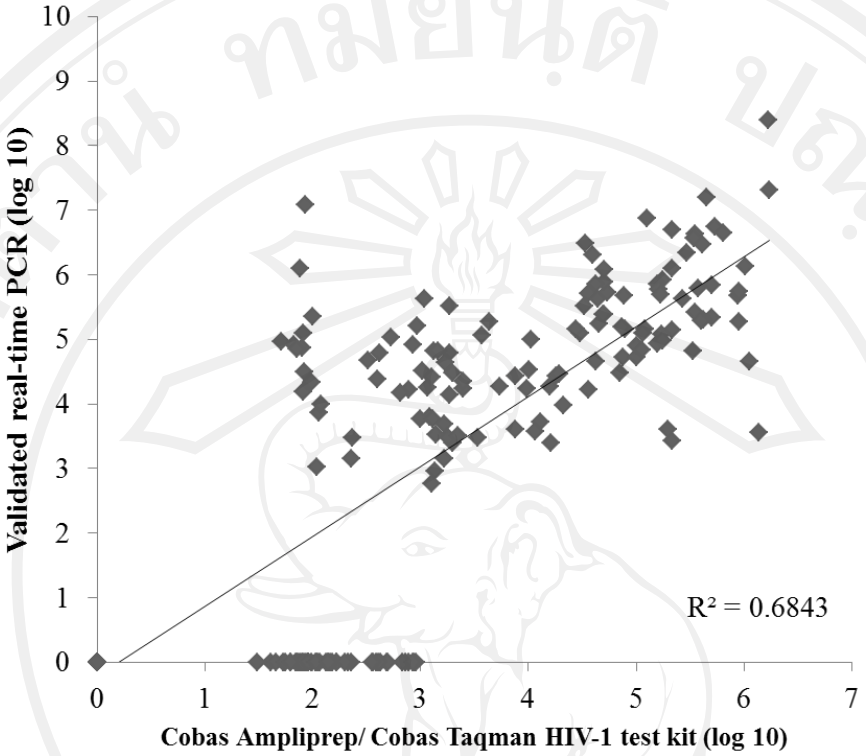

Figure 19 The correlation analysis of validated and reference methods.

The log10 of individual plasma viral load examined by developed assay (y-axis) and reference kit, CobasAmpliprep/ CobasTaqman HIV-1 test kit (x-axis) was plotted and the correlation coefficient value (r^2) of all plasma samples analyzed was calculated to be 0.6843.

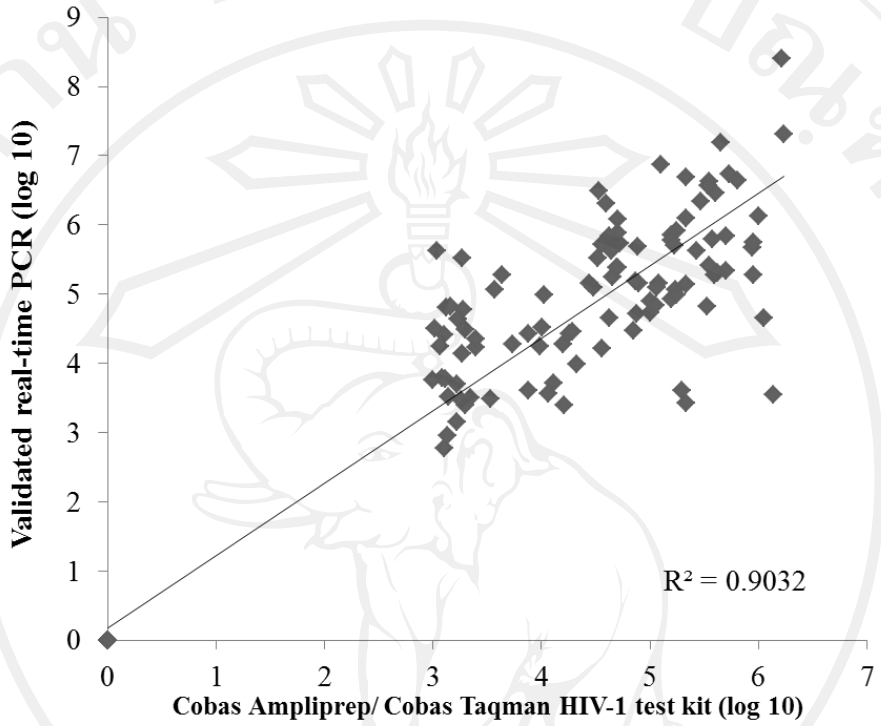

Figure 20 The correlation analysis of validated and reference methods.

The log10 of individual plasma viral load ranging from 10^3-1.7x10^6 copies/ml by developed assay (y- axis) and reference kit (x-axis) was plotted and the correlation coefficient value (r^2) of those plasma samples was calculated to be 0.9032.

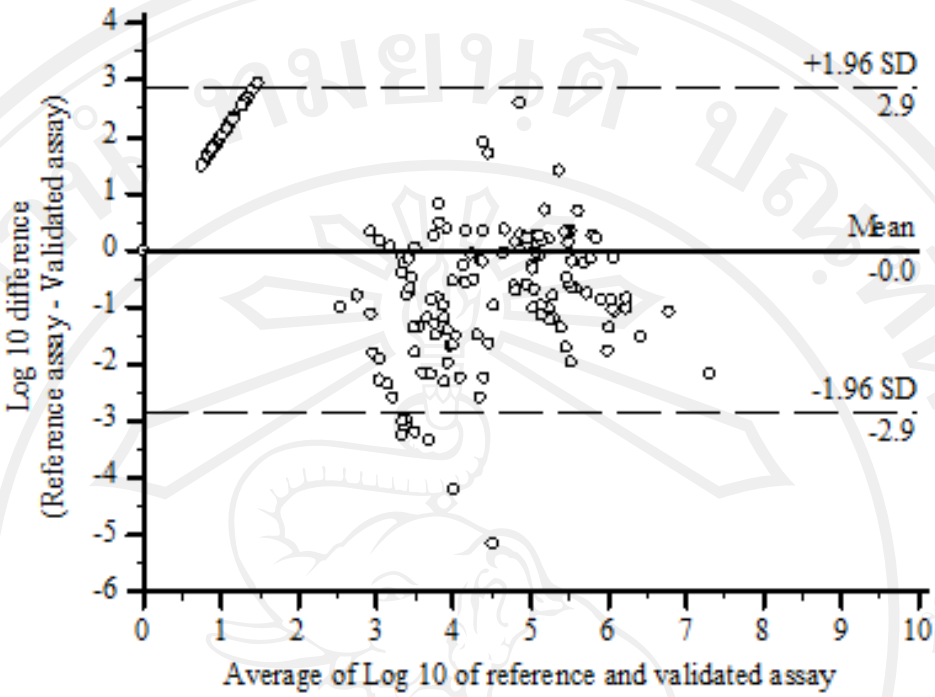

Figure 21 An agreement analysis of reference and validated assay.

Bland-Altman plot was applied for agreement analysis of reference and validated assay. The different of reference HIV-1 commercial kit and established real-time PCR (Y-axis) was plotted against the average in log10 of both assays (X-axis). The mean difference and standard deviations (SD) was shown in solid and dash line, respectively. The open circle indicated each sample that was determined in this study.