CHAPTER IV

RESULTS

1. HIV-1 RNA standard quantitation curve

The HIV-1 RNA standard quantitation curve was prepared by using HIV-1 RNA extracted from HIV-1 positive pool plasma. After pooling, 3 aliquots of pooled plasma were sampling and re-quantified by using Amplicor HIV-1 Monitor® version 1.5. The viral load results from each sample were 2.71×10^5, 4.25×10^5 and 4.71×10^5 copies/ml and the mean value was 3.89×10^5 copies/ml. To create the external standard quantitation curve, the pooled plasma was diluted with HIV-1 negative pool plasma from $1x10^2$ to$1x10^5$ copies/reaction (8 µl of extracted RNA) and processed for RNA extraction and amplification. Each dilution was repeated for five times. The fluorescent signal generated at the elongation step was collected and plotted against the cycle number (Figure 4; Upper). The external standard curve is a plot of cycle number at the crossing point (threshold cycle; C_T) versus the logarithm of initial template amount (copy number) using LightCycler™ software version 3.5 that coupled to the machine (Figure 4; Lower). In order to determine the C_T, the LightCycler™ software provides two analysis algorithms; Fit points method and Second derivative maximum method. For the Fit point method, the user determines the baseline adjustment, noise band setting, crossing line setting and choice of fit points. This manual selection of parameters may allow variation occurs between persons and runs. In contrast, the Second derivative maximum method is an automatic calculation and allows no user input. It offers the advantages of speed and simplicity, especially for quick analysis of frequently repeated standard curves. So, we decided to determine C_T by the Second derivative maximum method throughout the study.

By this software, the key parameters used to determine the performance of regression line such as slope, error and regression coefficient (r) were also considered.

The slope which defines the distance between data points on the regression line can be converted into efficiency of amplification (E) with the following formula:

$$E = 10^{-1/slope}$$

For the present standard curve shown in figure 4 given a slope value of -2.732, when calculated, the maximal efficiency of 2.32 was obtained. Its' mean that the amount of amplified product was double at each cycle or exponentially increase.

The error value of 0.0953 was within the limit of allowable value ($\leq$ 0.1) indicating the low variation between assay tube to tube due to pipetting errors.

The regression coefficient (r) is -0.99 which close to the allowable value of -1.0 also indicating well suitability of the linear fit (almost all samples points are found on the regression curve) due to the low systematic errors, e.g. error in dilution series. Taken together, the high efficiency of amplification, well linearity and low error value make the external standard curve performed in this study is successfully and accurately used to quantify of HIV-1 from unknown samples.

2. Reproducibility of assays

Reproducibility is a key parameter to consider in quantitative amplification. It determines the minimum difference in initial target concentration that the assay can distinguish. To evaluate the reproducibility of this assay, both intra- and inter-assay were performed using serial dilutions of HIV-1 positive pool plasma. The intra-assay was designed to test for the variation within the same run while the inter-assay tests for the variability between different runs. For intra-assay, the reproducibility was evaluated using five different HIV-1 concentrations; 10^5, $5x10^4$, 10^4, 10^3, and 10^2 copies/reactions, and run in five replicate each. The standard deviation (S.D.) and coefficient of variation (CV) of C_T were calculated and shown in table 7. The % CV of all dilutions tested was between 0.53-1.90. The lower percentage of CV indicated the less variation in the assay. However, the variability was significantly high especially, in the plasma with low copy number of HIV-1 RNA (%CV 1.9 at 10^2 copies/reactions).

For the inter-assay variation testing, four concentration of HIV RNA; 10^5, 10^4, 10^3 and 10^2 copies/reaction were used and repeated for ten times. The result was shown in table 8. The %CV was ranging from 3.42-6.17 which higher than those obtained from the intra-run assays.

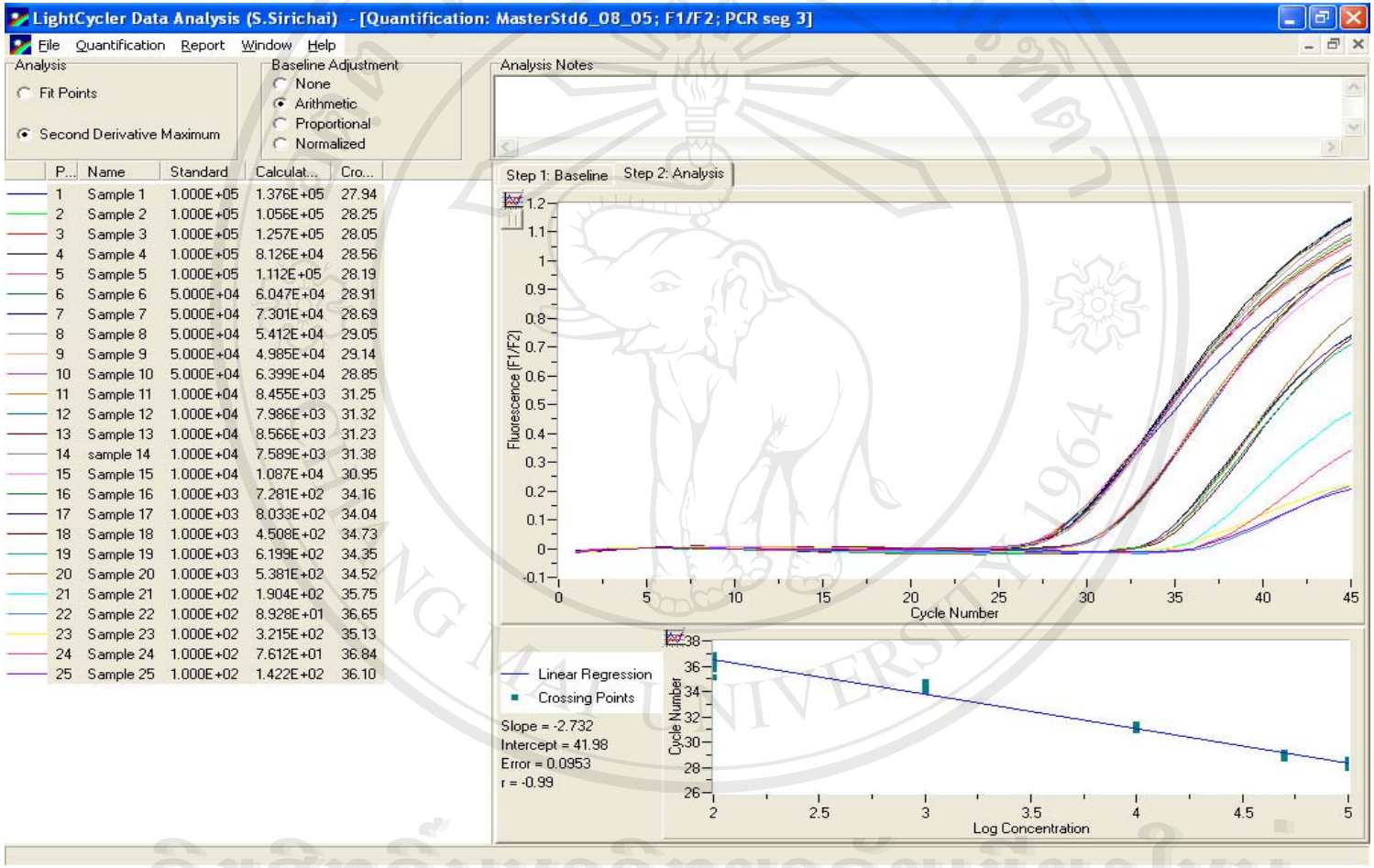

Figure 4. Illustration of the fluorescent signal curve obtained from intra assay of HIV-1 RNA dilutions by using real-time PCR on the LightCycler™ system; Upper is a plot between the fluorescent signal and cycle number of PCR; Lower is a plot between cycle threshold (C_T) and Log_{10} RNA copy number.

.**Table 7 Determination of assay precision by intra-assay variation analysis**

Copies/reaction	Cycle Threshold (C_T)							
	1	2	3	4	5	Mean	SD	%CV
1.00E+05	27.94	28.25	28.05	28.56	28.19	28.198	0.236	0.84
5.00E+04	28.91	28.69	29.05	29.14	28.85	28.928	0.175	0.61
1.00E+04	31.25	31.32	31.23	31.38	30.95	31.226	0.165	0.53
1.00E+03	34.16	34.04	34.73	34.35	34.52	34.360	0.276	0.80
1.00E+02	35.75	36.65	35.15	36.84	36.10	36.098	0.685	1.90

S.D. : Standard Deviation; CV: Coefficiency of Variation

Table 8 Determination of assay reproducibility by inter-assay variation analysis

Copies/reaction	Cycle Threshold (C_T)												
	1	2	3	4	5	6	7	8	9	10	Mean	SD	%CV
1.00E+05	28.30	27.96	30.24	31.11	29.42	31.55	28.25	26.07	27.28	27.41	28.759	1.773	6.17
1.00E+04	30.79	30.45	33.50	32.75	32.25	31.79	31.32	29.24	29.80	29.92	31.181	1.387	4.45
1.00E+03	35.85	35.71	35.66	36.09	34.58	36.26	34.04	33.62	32.59	32.64	34.704	1.412	4.07
1.00E+02	37.77	36.05	38.10	38.26	36.97	38.46	36.65	38.34	35.05	35.44	37.109	1.268	3.42

S.D. : Standard Deviation; CV: Coefficiency of Variation

3. Detection limit

To determine the detection limit of the assay, HIV-1 positive pool plasma was serially diluted from 1,000-50 copies/ml and quantify for RNA copy number using standard protocol (140 µl of plasma used) described in materials and methods. The assay was repeated ten times in all dilution tested. The result was shown in table 9. The positive fluorescent signal was detected in all runs (100%) of the assay at 1,000 copies/ml but not always detected at 500 copies/ml (80%). There was no fluorescent signal detected at 100 and 50 copies/ml. Thus, the detection limit of the standard protocol assay was therefore at 1,000 copies/ml which corresponds to 23 RNA copies/reaction.

Table 9 Detection Limit of HIV-1 RNA quantification by In-house quantitative real-time-PCR assay on LightCycler™ technology using 140 µl of plasma

Pool plasma standard serum dilution (copies/ml)	%Detection (n=10)	C_T mean value	S.D.	%CV
1,000	100 (10/10)	37.75	1.85	4.89
500	80 (8/10)	38.43	1.25	3.25
100	0(0/10)	ND	-	-
50	0(0/10)	ND	-	-

S.D. : Standard Deviation; CV: Coefficiency of Variation; ND: Not Detected

4. Increasing sensitivity of assay

Since the fluorescent signal vary directly to the number of the initial template. To increase the sensitivity of the assay, we therefore increased the plasma volume used for RNA extraction from 140 µl to 1 ml. Again, in order to save cost of the

extraction reagents, we concentrated the virus by centrifuging 1 ml of plasma at 23,000xg for 1 hr at 4ºC, then carefully aspirated and discarded approximately 860 µl of the upper part of plasma or until 140 µl of plasma left in the tube. The plasma was processed for RNA extraction and amplification by using standard protocols as described above. The results from table 10 showed that positive fluorescent signal was now detected in all runs (100%) at RNA 500 copies/ml with the mean C_T value of 38.0 and %CV 2.96. However, the assay could detect HIV-1 RNA as low as 100 and 50 copies/ml (corresponds to 16 and 8 copies/reaction respectively) at 90% and 70% respectively. When the same RNA extracts were re-assay using the same PCR reagents and analysis comparing with other instrument (Chromo 4, MJ research instrument, USA), the sample that was missed by the earlier assay was detected but with high C_T value. Hence, when increase the plasma volume to 1 ml, the detection limit of the assay was decreased 10 times from 1,000 to 100 copies/ml. Furthermore, it was interesting that at 50 copies/ml of RNA, this assay could detect up to 70%. However, beside from increasing the plasma volume, the other strategy to further increase the sensitivity or lowering the limit of detection was to increase the volume of RNA extract in the reaction. Unfortunately, the LightCycler™ technology allows using only 20 µl of total reaction volume, thus, maximally only 8 µl of RNA extract could be added in each reaction. This was another limitation dependent on the instrument.

Table 10 Detection Limit of HIV-1 RNA quantification by In-house quantitative real-time-PCR assay on LightCycler™ system using 1 ml of plasma

Pool plasma HIV-1 standard dilution (copies/ml)	%Detection (n=10)	C_T mean value	S.D.	%CV
500	100 (10/10)	38.00	1.13	2.96
100	90 (9/10)	40.11	1.26	3.14
50	70 (7/10)	40.22	1.47	3.66

S.D. : Standard Deviation; CV: Coefficient of Variation; ND: Not Detected

5. Quantification with the external master standard curve

Most absolute quantification of target viral RNA require the use of external standard curve generated from known concentration of standard RNA. Both standard RNA and unknown samples have to perform each time on the same run. Unavoidable, this is an extra-cost added directly to the assay cost and also limited the space for running. For example, using LightCycler™ instrument, the space or capacity of running for each assay is limited at 32 samples, subtraction of 4 samples for the standard RNA, it is going to be only 28 available for the unknown samples. However, the LightCycler™ software version 3.5 or higher allows reuse of standard curve which has been generated in a previous runs. This especially suitable for applications where the same parameter is analyzed in multiple runs.

We therefore, evaluated the application of master standard curve for absolute quantitation of HIV-1 in our study. HIV-1 positive pooled plasma was diluted until got the final concentration at 50, 100, 500 and 1,000 copies/ml of RNA and processed for RNA extraction and amplification along with four concentrations of standard RNA (1×10^5, 1×10^4, 1×10^3 and 1×10^2 copies/reaction). Each concentration of samples was repeated 10 times. The absolute copy number of RNA was calculated using its' own standard curve and the imported master standard curve performed in this study and plot the linear regression curve. The result in figure 5 showed a good correlation between the results obtained from 2 methods of analysis with the r value of 0.9203. This result indicating that the master standard curve system can be alternatively applied to the assay format developed in this study.

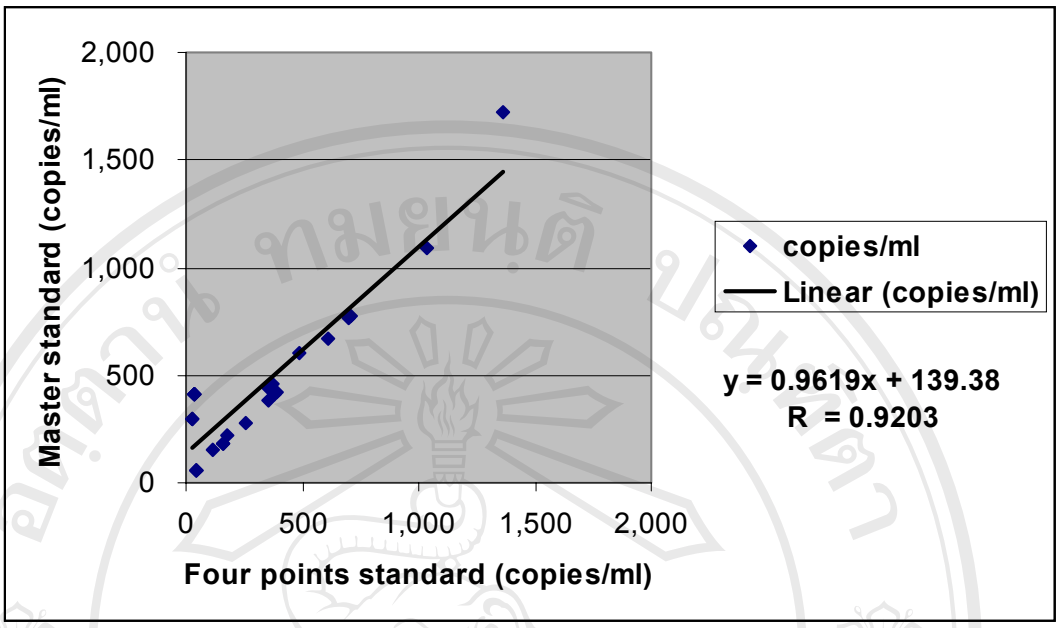

Figure 5. Correlation between absolute copy number of HIV-1 RNA calculated by using external standard curve ran in parallel with the sample and external master standard curve from the previous run

6. Comparison of plasma viral load quantitation by using In-house quantitative real-time PCR and Amplicor®HIV-1 Monitor assay Kit

Ninety six clinical samples tested by using Amplicor HIV-1 Monitor® assay kit with viral load ranging from > 750,000 to <50 copies/ml were collected and re-quantified for HIV-1 viral load by using in-house quantitative real-time PCR developed in this study. Ten plasma samples from healthy blood donors were also included in the assay. The standard assay protocol with plasma volume 140 µl was used for RNA extraction and amplification. External standard curve was performed in parallel for each run. Both absolute copy number and its' logarithmic value of HIV-1 RNA quantified by in-house quantitative real-time PCR and Amplicor HIV-1 Monitor® assay were shown in table 11. Among 96 plasma samples, the Amplicor HIV-1 Monitor® assay could be detected the viral load value in 91samples while the in-house assay detected only in 87 samples.

Table 11 Cross sectional data of HIV-1 viral load result by using Amplicor HIV-1 Monitor® assay Kit and In-house real-time RT-PCR

	Amplicor		In-house RT-PCR	
N0	copies/ml	Log10	copies/ml	Log10
1	>750000	>5.87	33,963	4.53
2	>750000	>5.87	10,981	4.04
3	>750000	>5.87	19,181	4.28
4	>750000	>5.87	2,620,475	6.42
5	>750000	>5.87	2,216,500	6.35
6	>750000	>5.87	2,620,475	6.42
7	>750000	>5.87	2,216,500	6.35
8	863,000	5.94	75,598	4.88
9	896,000	5.95	923,450	5.97
10	622,608	5.79	22,575	4.35
11	616,000	5.79	794,200	5.90
12	475,181	5.68	804,650	5.91
13	446,000	5.65	52,993	4.72
14	277,000	5.44	74,030	4.87
15	264,000	5.42	450,725	5.65
16	262,000	5.42	51,205	4.71
17	253,366	5.40	750,200	5.88
18	247,813	5.39	96,883	4.99
19	241,879	5.38	48,895	4.69
20	197,538	5.30	30,828	4.49
21	190,614	5.28	61,298	4.79
22	188,000	5.27	104,418	5.02
23	172,414	5.24	16,860	4.23
24	137,000	5.14	13,285	4.12
25	104,000	5.02	114,785	5.06
26	99,424	5.00	155,128	5.19
27	98,460	4.99	175,808	5.25
28	95,300	4.98	ND	ND
29	76,538	4.88	ND	ND
30	67,444	4.83	30,690	4.49
31	67,000	4.83	28,105	4.45
32	63,648	4.80	44,495	4.65
33	56,472	4.75	34,705	4.54
34	54,300	4.73	15,045	4.18
35	51,483	4.71	388,575	5.59
36	45,911	4.66	70,730	4.85
37	43,160	4.64	13,310	4.12
38	42,188	4.63	25,440	4.41
39	41,723	4.62	7,332	3.87
40	40,653	4.61	10,225	4.01
41	39,400	4.60	32,258	4.51
42	39,000	4.59	41,525	4.62

Table 11 Cross sectional data of HIV-1 viral load result by using Amplicor HIV-1 Monitor® assay Kit and In-house real-time RT-PCR (Continue)

	Amplicor		In-house RT-PCR	
N0	copies/ml	Log10	copies/ml	Log10
43	39,936	4.60	429,825	5.63
44	38,319	4.58	13,098	4.12
45	37,200	4.57	47,768	4.68
46	32,565	4.51	9,620	3.98
47	28,907	4.46	3,196	3.50
48	24,286	4.39	5,132	3.71
49	23,400	4.37	22,952	4.36
50	23,074	4.36	5,935	3.77
51	19,700	4.29	16,426	4.22
52	18,456	4.27	5,998	3.78
53	17,600	4.25	14,234	4.15
54	16,533	4.22	16,440	4.22
55	15,148	4.18	7,714	3.89
56	14,600	4.16	9,446	3.98
57	13,956	4.14	1,543	3.19
58	10,860	4.04	4,345	3.64
59	9,664	3.99	1700	3.23
60	9,172	3.96	3,542	3.55
61	7,760	3.89	4,777	3.68
62	7,303	3.86	4,549	3.66
63	6,530	3.81	4,169	3.62
64	6,178	3.79	8,434	3.93
65	6,000	3.78	1,956	3.29
66	5,814	3.76	4,568	3.66
67	5,478	3.74	170	2.23
68	5,460	3.74	2,468	3.39
69	5,251	3.72	1,454	3.16
70	4,927	3.69	416,350	5.62
71	4,845	3.69	5,077	3.71
72	4,395	3.64	4,659	3.67
73	3,770	3.58	2,943	3.47
74	3,620	3.56	9,433	3.97
75	2,552	3.41	ND	ND
76	2,285	3.36	1,126	3.05
77	2,071	3.32	335	2.52
78	1,840	3.26	561	2.75
79	1,730	3.24	6,009	3.78
80	1,525	3.18	947	2.98
81	1,560	3.19	618	2.79
82	1,520	3.18	ND	ND
83	1,352	3.13	356,675	5.55

Table 11 Cross sectional data of HIV-1 viral load result by using Amplicor HIV-1 Monitor® assay Kit and In-house real-time RT-PCR (Continue)

	Amplicor		In-house RT-PCR	
N0	copies/ml	Log10	copies/ml	Log10
84	1,043	3.02	256	2.41
85	557	2.75	570	2.76
86	428	2.63	209	2.32
87	332	2.52	109	2.04
88	259	2.41	97	1.98
89	255	2.41	54	1.73
90	252	2.40	ND	ND
91	245	2.39	40	1.60
92	184	2.26	ND	ND
93	160	2.20	ND	ND
94	148	2.17	229	2.36
95	79	1.90	ND	ND
96	53	1.72	110	2.04
97	36	1.56	72	1.85
98	<50	<1.7	ND	ND
99	<50	<1.7	ND	ND
100	<50	<1.7	ND	ND
101	<50	<1.7	ND	ND
102	<50	<1.7	ND	ND

ND; not detected

Among those undetected samples, four with low viral load (79-252 copies/ml) by Amplicor HIV-1 Monitor® assay were not detected by the in-house assay due to the detection limit. Whereas, the other five have been classified as samples carrying HIV-1 RNA <50 copies/ml or not detected due to the limit of quantification by Amplicor HIV-1 Monitor® assay were also not detected by the in-house assay. In addition, the positive fluorescent signal was not detected in all plasma from healthy blood donors, indicating also the specificity of the assay.

However, it was interesting that, among seven samples with high viral load (> 750,000 copies/ml; maximum limit of the test) by Amplicor HIV-1 Monitor® assay, four samples were still detected by the in-house assay with the viral load value between 2.2-2.6x10^6 copies/ml. This result indicating that the in-house assay has the upper limit of detection at least 1 $\log_{10}$ higher than the Amplicor HIV-1 Monitor®

assay (>750,000 vs. > 2.6 x10^6 copies/ml). The explanation for the other three samples where the in-house assay detected the viral load almost 2 $\log_{10}$ lower than the Amplicor HIV-1 Monitor® assay may due to the condition and long term of storage the samples.

However, when $\log_{10}$ value of RNA copy number obtained from both techniques were linear plotted, a highly correlation was observed (r = 0.899) (Figure 6).

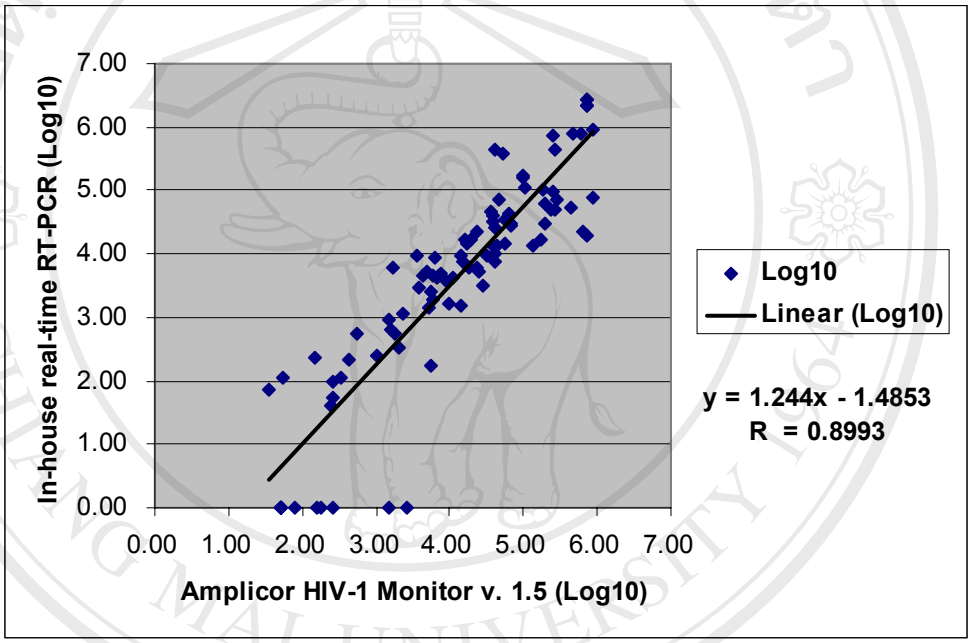

Figure 6 Correlation between $\log_{10}$ HIV-1 RNA copy number from clinical samples as measured by the Amplicor®HIV-1 Monitor assay Kit version 1.5 and In-house quantitative real-time PCR.

Moreover, the high correlation value was found especially at log 3-5 or between 10^3-10^5 RNA copies/ml. The absolute copy number and $\log_{10}$ values were also comparatively analyzed with the master standard curve. The $\log_{10}$ values obtained from both types of analysis curve were linear plotted (Figure 7). The high correlation was observed (r= 0.988) indicating that the master standard curve can efficiently be the alternative low cost system used to quantify the HIV-1 RNA in clinical plasma samples.

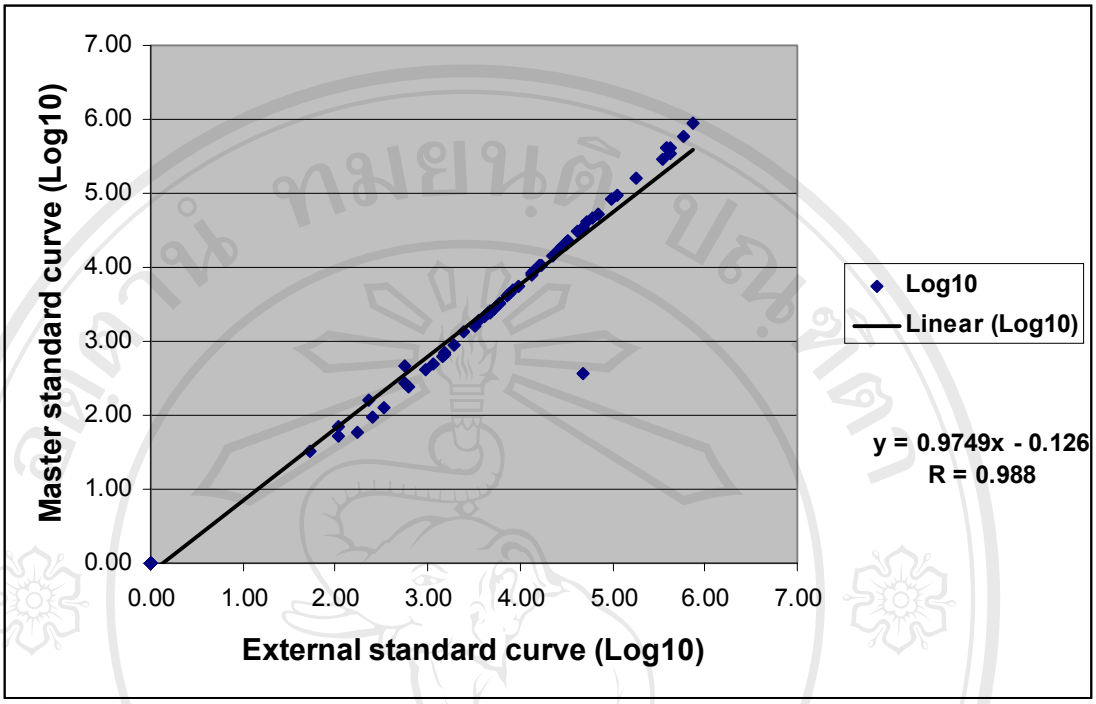

Figure 7 Correlation between Log10 HIV-1 RNA copy number in clinical samples determined by using In-house quantitation real-time PCR and analyzed by using external standard curve ran in parallel with the sample and external master standard curve from the previous run

7. Cost Analysis of in-house quantitative real-time PCR on LightCycler® for HIV-1 viral load assay

The assay cost was analyzed based mainly on the assay procedures; RNA extraction, amplification and detection. The cost of instruments, other hardware or service contract was not included. The labor cost which is highly varied among institutions was also not included for calculation. Cost of all reagents and consumables were based on price list on October 2005 and no discount pricing was used for analysis. Cost of HIV-1 viral load assay in Thailand was varied widely with the company and number of purchasing. For example, the price of Amplicor HIV-1

Monitor® version 1.5 from Roche Diagnostic Inc., USA which has a biggest market shared in Thailand is varies from 2,000 to 3,300 baht/test (consumables not included) depending on purchasing number. In this study, we also compared the cost of our assay with Amplicor HIV-1 Monitor® version 1.5. The results in table 12 and 13 revealed that, including all consumables and reagents, cost of the in-house assay was 5-7 times (410 vs. 2,140-3,140 Baht) lower than Amplicor HIV-1 Monitor® version 1.5. The home-brew reagent mix which all the reagent were brought separately; dNTP, reverse transcriptase and *Taq* DNA polymerase, was less expensive than the ready mastermix or with other enzyme mix. The home-brew reagent cost around 55-142 Baht depend on quality of enzyme used; AMV, Omniscript or Sensiscript reverse transcriptase while the QuantiTech Probe RT-PCR reagent mix used in this study cost about 100 Baht per reaction. However, using home-brew reagent mix has some drawback in quality control; variability in reagent used with no manufacturer's quality assurance or technical error in mixing the reagents. At present, there are various kinds of reagent mix produced from different companies for different kinds of machines, the cost are varies from 80 to 500 Baht. Comparative cost analysis between the reagent mix was shown in table 14.

The hand-on time was also analyzed depending on the steps of the assay. Since our in-house assay is real-time based technology, the amplification and detection are occur in the same time, thus, the assay time is generally less than any standard PCR. For full scale running (32 capillaries) on the LightCycler™, the viral load result of 28 clinical samples plus 4 capillaries for standard RNA could be obtained within 2 hours. Comparison to the commercially available test kits, the total assay time of Roche Amplicor HIV-1 Monitor® v 1.5 assay Kit and bDNA (Bayer, USA) assay were close to 6 hours with the same number of samples. Thus, our in-house assay developed in this study has lower cost and less hand-on time compared to the commercially available test kits.

TABLE 12 Comparison of cost and hand-on time between In-house quantitative real-time PCR and Amplicor™ HIV-1 Monitor 1.5 assay Kit for quantitation of HIV-1 RNA

Cost category	In-house Quantitative Assay (Bath)	Amplicor™ 1.5 (Bath)
Cost per assay		
Sample preparation (RNA extraction reagent)	190	-
RT-PCR reagent mix	100	2,000-3,000
Primers and TaqMan Probe	25	-
Comsumables		
• Capillary	30	-
• Other materials and reagent; filter tips, transfer pipettes, micro-tubes, gloves, ethanol	65	140
Total cost	**410**	**2,140-3,140**
Hand-on time		
Specimen preparation	20 min	45 min
Amplification&Detection	105 min	-
Amplification	-	120 min
Detection	-	120 min
Total hand-on time	**125 min**	**285 min**

Table 13 Estimated consumables cost of In-house quantitative real-time PCR and Amplicor HIV-1 Monitor® 1.5 assay Kit

Consumables	Cost/piece (Bath)	Number used		In-house assay	Amplicor
		In-house assay	Amplicor		
Filtered tips 200 ul	5	2	12	10	60
Filtered tip 1000 ul	4	6	6	24	24
Filtered tips 20 ul	5	3	7	12	35
Transfer pipette	1	-	2	0	2
Micro-tube	1	2	2	2	2
Glove	1	5	5	5	5
Other reagents; Plastic tube 15 ml alcohol, water		10	12	10	12
Total cost				**65**	**140**

Table 14 Cost of Home-brew reagent and commercial reagent kits for one-step RT-PCR (20ul)

	Home-brew	Omniscript (50 ng-2 ug RNA)	Sensisript (<50 ng RNA)	Quantitect -Hotstar Taq	Superscript III platinumTaq
dNTP(300uM each)	3	-	-	-	-
RT (25U)	35	101	107	-	-
Taq 1U	15-35	15-35	15-35	-	-
Total cost	53-73	116-136	122-142	100	75