CHAPTER V

RESULTS

Part I Development of the HIV-1 integrase RNA templates to generate the standard curve and positive control.

An important issue of in-house HIV-RNA quantitative is a reliable standard curve. Standard HIV-1 RNA was produced by cloning of whole HIV-1 integrase gene into pCI plasmid vector with T7 transcription site. The pCI-HIV-1 integrase plasmids were then transcripted to 386 bp RNA which was later used as standard HIV-RNA. The standard HIV-RNA was quantitated by RiboGreen RNA Quantitation Assay. (Molecular Probe, Roche Molecular System, Inc. Branchburg, NJ, USA.) The purity of HIV-1 integrase RNA template was obtained as shown in Figure 11. PCR without reverse transcription step was also tested to ensure complete of DNase digestion (Figure 12).

PART II Development of One-Step RT Real-time PCR for Quantification of HIV-1 integrase RNA.

To get the best efficiency of the One-Step RT Real-Time PCR assay, different primer and probe concentrations and hybridization temperatures were evaluated. The most optimized and cost-effective parameters were obtained as described in the material and methods section. The concentration of HIV-1 integrase RNA was quantified by RiboGreen RNA Quantitation Assay. The HIV-1 integrase RNA construct was diluted 10- fold serially at 5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 copies/reaction and used for generating an external standard curve at each run. The amplification plots of a 10-fold serially diluted calibrator HIV-RNA is shown in Figure 13a. A regression curve obtained term the threshold cycle (C_t) values *versus* $\log_{10}$ concentrations of a standard HIV-1 integrase RNA plot has showed a highly correlation (r^2 = 0.97, efficiency = 89%) (Figure 13b). The percent amplification efficiency was calculated using this formula: $(10^{(-1/slope)}-1)\times100$.

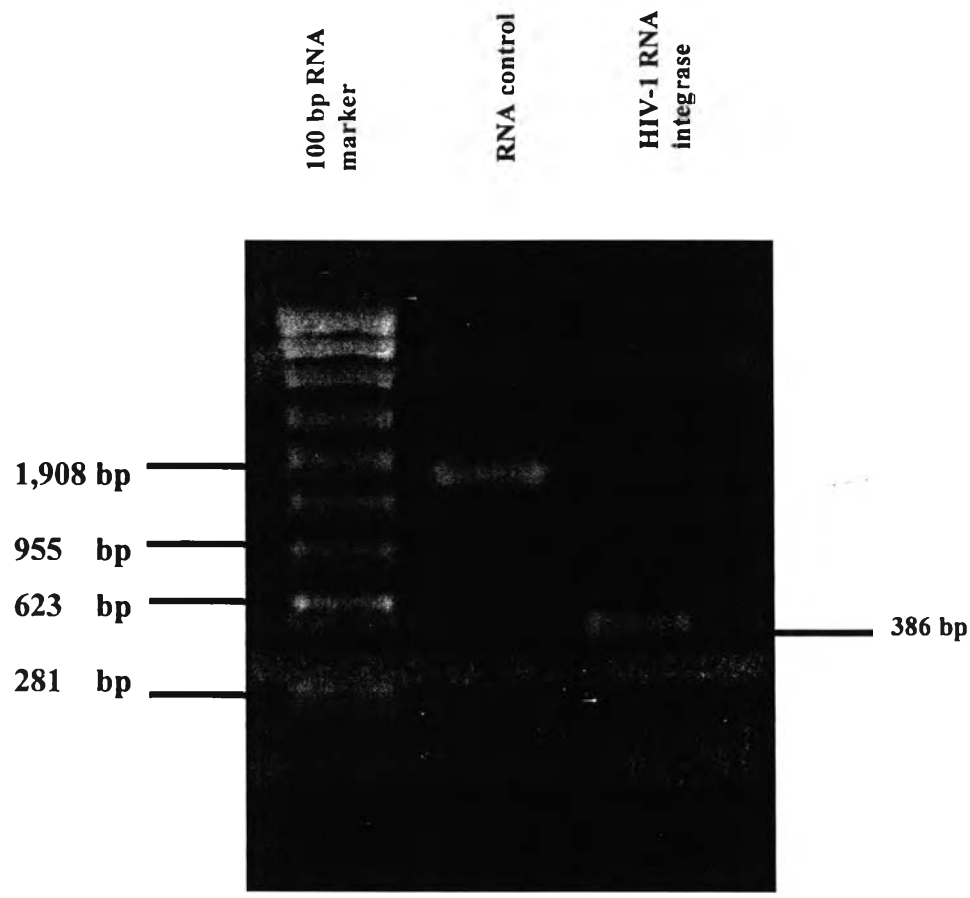


Figure 11 High quality of transcripted 386 bp HIV-1 integrase RNA was obtained to be used for the standard curve generation when performing the real-time PCR assay.

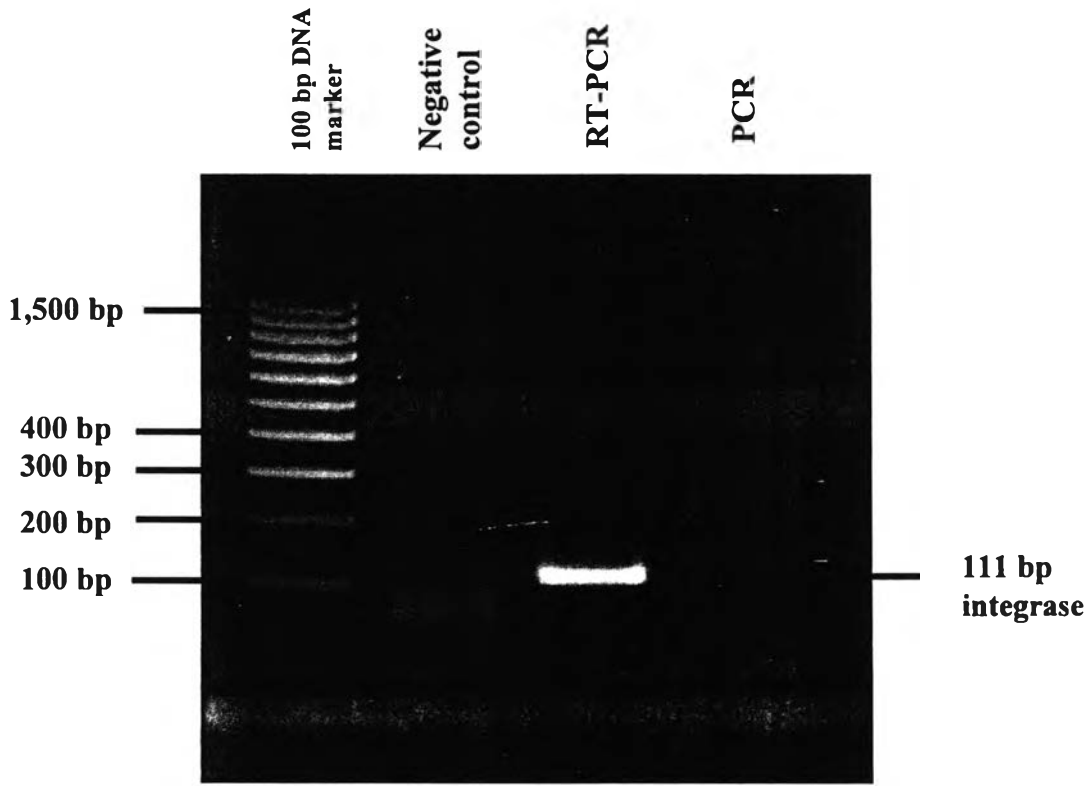
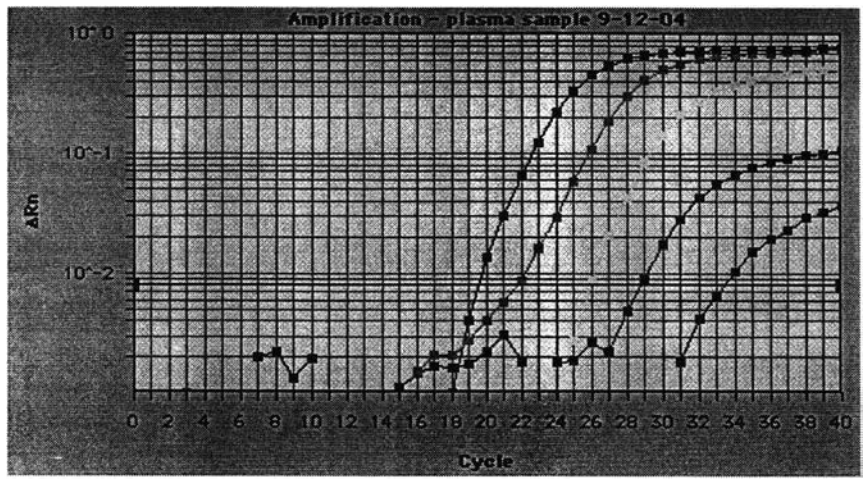


Figure 12 The result confirmed that there was no DNA contamination in the *in vitro* transcripted HIV-1 integrase RNA. As seen by only lane 3 of which DNase pretreated sample when RT-PCR was performed, showed a positive 111 bp of RT-PCR product. In contrast in lane 4 of which direct PCR without RT step was done, there was no the product observed.

13a



13b

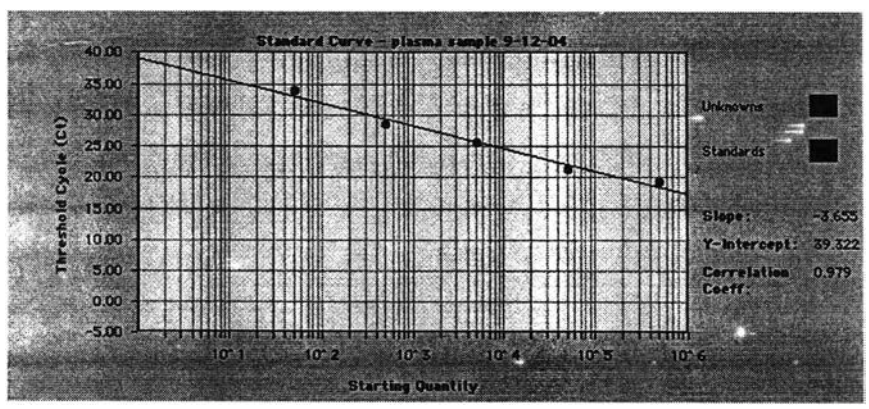


Figure 13: (a) Amplification plot of 10-fold serial dilutions of the transcripted HIV-1 integrase RNA as a standard curve for HIV-1 RNA measurement by One-Step RT Real-Time PCR. The amounts of input RNA were 5×10^5, 5×10^4, 5×10^3, 5×10^2 and 50 copies/reaction. (b)The standard curve was expressed as Ct value versus viral RNA copies/reaction as described in the material and methods.

PART III Assay validation

Analytical specificity, sensitivity and reproducibility.

To test the specificity of the assay, 25 HIV-1 negative plasma samples were tested. The result was shown in Table 6. Three of 25 samples were positive by One- Step RT Real-Time PCR. The viral load results are 11,826, 2,774 and 468 copies/ml. All of these samples were repeated, 1 sample still showed a result with HIV-1 RNA viral load is 462 copies/ml. A conventional multiplex nested PCR in *gag* and *pol* region had shown a negative result.

To evaluate the sensitivity of the assay, several serial dilutions of the HIV-RNA standard starting from 5×10^5 to 5 copies/ml were tested. When 5 copies of RNA was introduced into the reaction, the detection failed in most case whereas 4 out 5 (80%) of 50 copies/ml were positive and 500 copies/ml were always detectable (100%).(Table 8)

In order to exclude the possible presence of residual PCR inhibitor after extraction, 10-fold serially dilutions of HIV-1_{IIIB} (obtained from $MT4_{IIIB}$ cell lines) spiked in HIV-negative plasma from 5×10^5 to 50 copies/ml were tested. Three out of 4 (75%) of 50 copies/ml were positives and 500 copies/ml were always detectable (100%). Hence the detection limit of our technique was set at 500 copies/ml. (Table 9)

To evaluate the variability between different runs. The reproducibility of the technique both intra-assay and inter-assay variations were performed. The intra-assay coefficient of variation was evaluated by testing 10 replicates of 5 different dilutions of HIV-RNA standard between 5×10^5 to 50 copies/ml each repeated 5 times. Intra-assay coefficients of variation are presented in Table 7. The coefficient of variation (CV) of C_t was $\leq$ 5% for all of the standard HIV-1 RNA dilutions tested. The inter-assay reproducibility was obtained five different experiments. The results are shown in Table 8. indicating the CV of < 6% for all of the standard HIV-RNA dilutions. The reproducibility of the assay was also analyzed using 4 replicates of 5 different dilutions of HIV-1_{IIIB} spiked in HIV-negative plasma varying between 5×10^5 to 50 copies/ml. The assay was repeated 2 times. The results are shown in Table 9 and Table 10. The coefficient of variation (CV) of Ct was < 7% for all of the dilutions tested.

Table 6 Viral load of 25 HIV–1 negative samples.

Patient	One-Step RT Real-Time PCR		HIV-1 antibody[a]
	1st	2nd	
1	<50	<50	Negative
2	<50	<50	Negative
3	<50	<50	Negative
4	<50	<50	Negative
5	11,826	<50	Negative
6	<50	<50	Negative
7	<50	<50	Negative
8	<50	<50	Negative
9	<50	<50	Negative
10	2,774	<50	Negative
11	<50	<50	Negative
12	<50	<50	Negative
13	<50	<50	Negative
14	468	462*	Negative
15	<50	<50	Negative
16	<50	<50	Negative
17	<50	<50	Negative
18	<50	<50	Negative
19	<50	<50	Negative
20	<50	<50	Negative
21	<50	<50	Negative
22	<50	<50	Negative
23	<50	<50	Negative
24	<50	<50	Negative
25	<50	<50	Negative

[a] The test were performed at the Anonymous Clinic, Thai Red Cross AIDS Research centre.

* Negative by conventional RT-PCR

Table 7 Comparison of the threshold cycle (C_t) values of standard curves and the results of intra-assay analyses.

In vitro transcripted HIV-1 integrase RNA (copies/ml)	Replicate										Mean	S.D.	%CV
	1	2	3	4	5	6	7	8	9	10			
500,000	18.09	17.66	18.03	18.03	18.05	18.05	17.8	18.01	18.07	17.86	17.97	0.15	0.82
50,000	20.99	21.13	21.07	21.04	21.15	21.15	21.06	21.24	21.41	21.17	21.19	0.21	1.00
5,000	24.91	24.44	24.45	24.11	24.27	24.39	24	24.55	24.45	24.25	24.38	0.25	1.03
500	26.62	26.31	26.62	26.72	26.39	23.77	26.96	25.17	NA	NA	26.02	1.18	4.57
50	29.41	29.63	29.16	32.8	31.32	NA	NA	NA	NA	NA	30.46	1.55	5.11

NA: not applicable; S.D: standard deviation; CV.: coefficient of variation

Table 8 Comparisons of the threshold cycle (C_t) values of standard curves and the results of inter-assay analyses.

In vitro transcripted HIV-1 integrase RNA (copies/ml)	Run					Mean	S.D.	%CV
	1	2	3	4	5			
500,000	19.25	19.61	21.39	19.20	21.20	20.13	1.07	5.31
50,000	22.15	23.61	24.08	21.10	24.23	23.03	1.35	5.86
5,000	27.16	28.29	29.91	29.16	29.13	28.82	1.11	3.85
500	32.90	33.62	32.59	32.39	32.90	32.88	0.46	1.39
50	36.40	Neg.	34.32	35.27	36.42	35.60	1.00	2.80

NA: not applicable; S.D: standard deviation; CV.: coefficient of variation

Table 9 Intra-assay validation (4 replicates) results of serial 10-fold dilution of HIV-1 $_{IIIB}$ viral particles.

HIV viral particle dilution (copies/ml)	Replicated				Mean	S.D.	%CV
	1	2	3	4			
500,000	231,433	731,975	427,432	401,653	448,123	208,257	3.70
50,000	55,399	71,143	137,134	85,030	87,177	35,436	2.70
5,000	2,940	2,579	1,456	1,607	2,146	741	2.00
500	218	145	87	34	121	78.97	3.70
50	66	25	15	Neg.	26.5	28.26	4.30

Neg.: Negative result; S.D: standard deviation; CV.: coefficient of variation.

Table 10 Inter-assay validation (2 runs) results of serial 10-fold dilution of HIV-1 $_{IIIB}$ viral particles.

HIV viral particle dilution (copies/ml)	Run		Mean	S.D.	%CV
	1	2			
500,000	383,109	448,123	415,616	45,971	5.00
50,000	19,175	87,177	53,176	48,084	0.04
5,000	1,277	2,146	1,711.50	614.47	3.40
500	136	121	128.50	10.60	6.30
50	10	35	22.50	17.67	5.80

S.D : standard deviation; CV.: coefficient of variation.

PART IV. Comparative of plasma HIV-1 quantitation using Bayer® HIV-1 RNA 3.0 Assay (bDNA) versus One-Step RT Real-Time PCR assay in HIV-1 seropositive patients.

Plasma samples were collected from 105 HIV-1 seropositive patients at the Anonymous Clinic, Thai Red Cross AIDS Research center. Two 1 ml aliquots of RNA extraction was performed from each of the samples. The in-house One-Step RT Real-Time PCR was performed in parallel in comparison to Bayer® HIV-1 RNA 3.0 Assay (bDNA). The results are summarized in Table 11. Median plasma HIV-1 RNA of The Bayer® HIV-1 RNA 3.0 Assay (bDNA) is 958 copies/ml as compared to that of the in-house One-Step Real-Time RT-PCR Assay is 362 copies/ml.(Figure 14) There is statistical difference (p = 0.024, Wilcoxon Signed Ranks Test)

As shown in Figure 15, the HIV-1 RNA quantification values obtained from the two assays were significantly correlated, as indicated by the Pearson correlation coefficient (r value) of 0.787 (p = 0.01). The correlation between the assays is good and significant in the plasma samples that showed HIV-1 RNA level $\geq$ 500 copies/ml by Bayer® HIV-1 RNA 3.0 Assay (bDNA) (r = 0.76, p = 0.01), but those with value < 500 copies/ml is non significant (r = 0.11, p = 0.21) (Table 12).

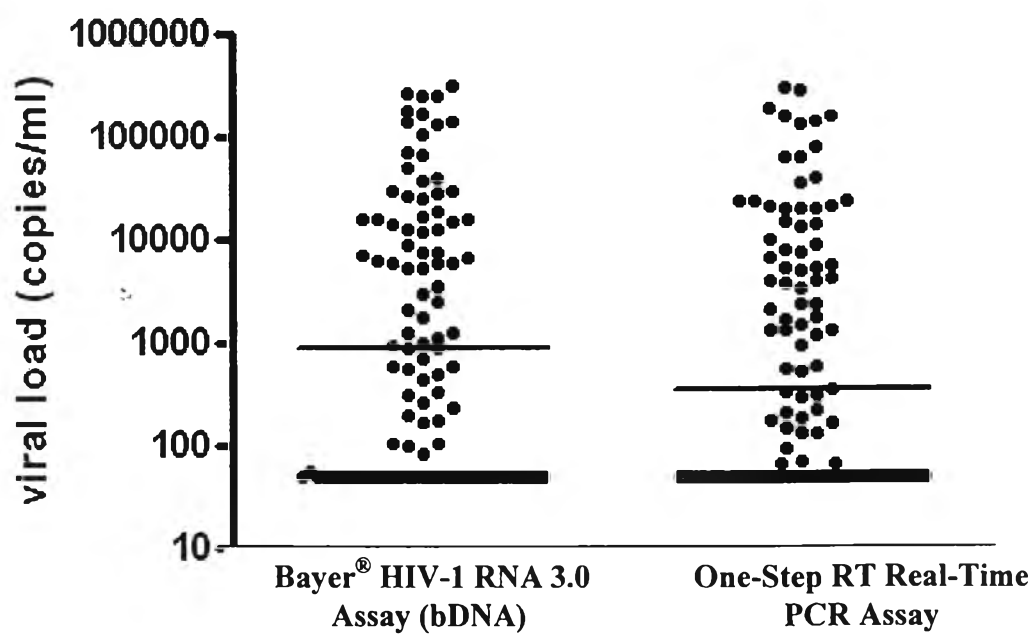


Figure 14 Median of plasma HIV-1 RNA (copies/ml) determined by Bayer® HIV-1 RNA 3.0 Assay (bDNA) and One-Step RT Real-Time PCR Assay is shown as solid lines. Median of Bayer® HIV-1 RNA 3.0 Assay (bDNA) is 985 copies/ml and mean of in house One-Step RT Real-Time PCR is 362 copies/ml.

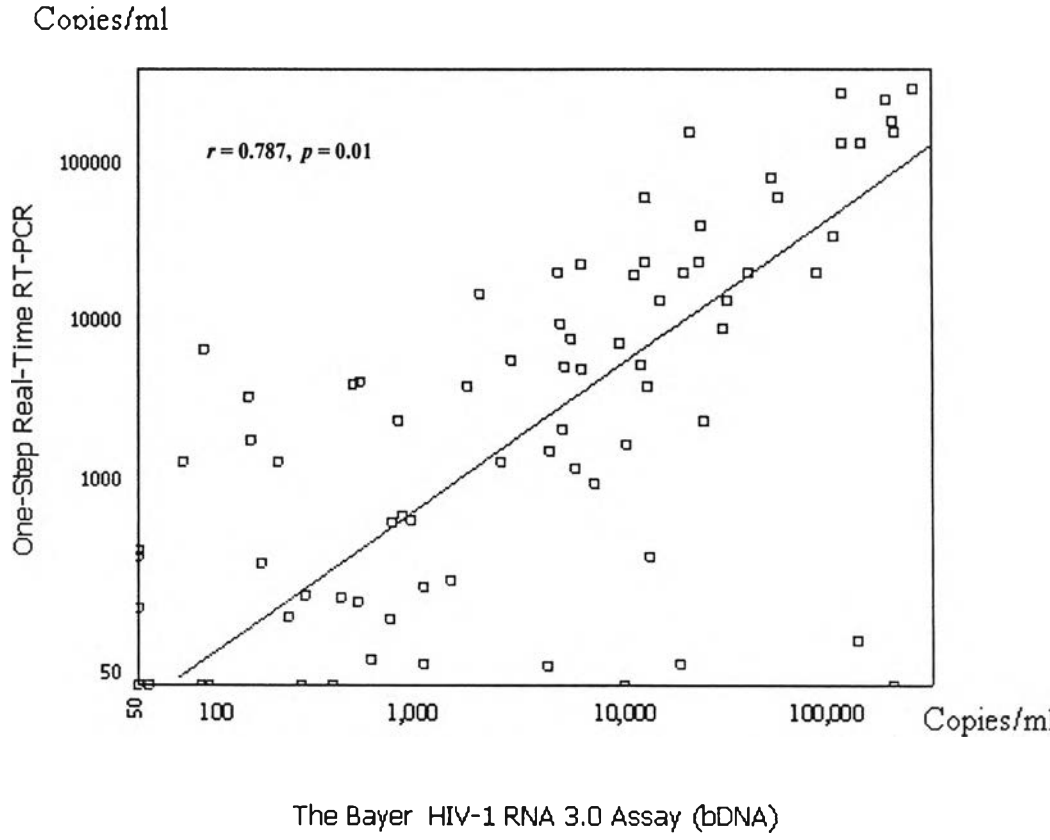


Figure 15 Scatter plot of plasma HIV-1 RNA copies/ml determined by Bayer® HIV-1 RNA 3.0 Assay (bDNA) and in house One-Step RT Real-Time PCR of 105 HIV-1 seropositive samples. The correlation coefficient (r) is 0.789 (p = 0.01).

Table 11 Parallel testing of 105 plasma samples by using Bayer® HIV-1 RNA 3.0 Assay (bDNA) and in house One-Step RT Real-Time PCR assay

Samples number	Patients ID number	Viral Load				Log different (bDNA -Real Time)
		Bayer® HIV-1 RNA 3.0 Assay (bDNA)		One-Step RT Real-Time PCR assay		
		(copies/ml)	Log	(copies/ml)	Log	
1	10110	107,675	5.0	20,742	4.3	0.7
2	10199	322,722	5.3	305,775	5.4	0.1
3	10135	235,932	5.3	260501	5.4	0.1
4	10222	143,657	5.1	279,006	5.4	0.3
5	10230	251,133	5.3	186,696	5.2	0.1
6	10312	141,836	5.1	136,131	5.1	0
7	10316	176,434	5.2	139,596	5.1	0.1
8	10333	131,014	5.1	35,598	4.5	0.6
9	10359	261,473	5.4	160,830	5.2	0.2
10	10357	254,317	5.4	<50	<1.7	≥3.7
11	10202	167,611	5.2	96	1.9	3.3
12	10127	14,152	4.1	20,100	4.3	0.2
13	10130	29,077	4.4	24,600	4.3	0.1
14	10129	11,830	4.0	7,500	3.8	0.2
15	10197	39,841	4.6	13,989	4.1	0.5
16	10211	70,077	4.8	62,031	4.7	0.1
17	10244	16,255	4.2	3,963	3.5	0.7
18	10274	50,334	4.7	20,835	4.3	0.4
19	10295	26,486	4.4	162,775	5.2	0.8
20	10234	15,974	4.2	62,247	4.7	0.5
21	10271	14,989	4.1	5,409	3.7	0.4
22	10239	16,007	4.2	24,063	4.3	0.1
23	10249	37,214	4.5	9,255	3.9	0.6
24	10287	29,359	4.4	41,160	4.6	0.2
25	10250	12,691	4.1	1,665	3.2	0.9

Samples number	Patients ID number	Viral Load				Log different (bDNA -Real Time)
		Bayer® HIV-1 RNA 3.0 Assay (bDNA)		One-Step RT Real-Time PCR assay		
		(copies/ml)	Log	(copies/ml)	Log	
26	10272	24,644	4.3	20,580	4.3	0
27	10263	18,784	4.2	13,761	4.1	0.1
28	10108	65,621	4.8	81,975	4.9	0.1
29	10207	30,340	4.4	2,379	3.3	1.1
30	10227	16,803	4.2	327	2.5	1.7
31	10219	12,500	4.0	<50	<1.7	≥2.3
32	10201	23,263	4.3	69	1.8	2.5
33	10058	9,022	3.9	960	2.9	1
34	10080	6,807	3.8	7,800	3.8	0
35	10112	1,764	3.2	231	2.3	1.1
36	10166	6,101	3.7	9,900	3.9	0.2
37	10170	2,478	3.3	15,300	4.1	0.8
38	10171	7,784	3.8	24,000	4.3	0.5
39	10277	3,496	3.5	5,838	3.7	0.2
40	10278	2,118	3.3	3,889	3.5	0.2
41	10238	5,957	3.7	20,601	4.3	0.6
42	10255	6,185	3.7	2,103	3.3	0.4
43	10346	6,426	3.8	5,262	3.7	0.1
44	10338	1,023	3.0	594	2.7	0.3
45	10309	3,094	3.4	1,317	3.1	0.3
46	10300	1,123	3.0	561	2.7	0.3
47	10298	7,254	3.8	1,179	3.0	0.8
48	10379	1,297	3.1	213	2.3	0.8
49	10377	5,441	3.7	1,539	3.1	0.6
50	10381	7,787	3.8	5,076	3.7	0.1
51	10208	1,289	3.1	69	1.8	1.3
52	10086	5,242	3.7	66	1.8	1.9
53	10045	714	2.8	72	1.8	1

Samples number	Patients ID number	Viral Load				Log different (bDNA -Real Time)
		Bayer® HIV-1 RNA 3.0 Assay (bDNA)		One-Step RT Real-Time PCR assay		
		(copies/ml)	Log	(copies/ml)	Log	
54	10077	958	2.9	2,400	3.3	0.4
55	10216	880	2.9	132	2.1	0.8
56	10228	604	2.7	168	2.2	0.6
57	10251	616	2.7	4,194	3.6	1.1
58	10240	574	2.7	4,116	3.6	0.9
59	10370	898	2.9	546	2.7	0.2
60	10068	201	2.3	300	2.4	0.1
61	10071	176	2.2	1,770	3.2	1
62	100078	329	2.5	189	2.2	0.3
63	10282	242	2.3	1,310	3.1	0.8
64	10256	104	2.0	6,717	3.8	1.8
65	10265	172	2.2	3,420	3.5	1.3
66	10281	83	1.9	1,332	3.1	1.2
67	10312	276	2.4	138	2.1	0.3
68	10320	453	2.6	<50	<1.7	≥0.9
69	10319	57	1.7	<50	<1.7	0
70	10344	315	2.5	<50	<1.7	≥0.8
71	10362	109	2.0	<50	<1.7	≥0.3
72	10385	103	2.0	<50	<1.7	≥0.3
73	102386	497	2.7	180	2.2	0.5
74	10044	<50	<1.7	330	2.5	≤-0.8
75	10046	<50	<1.7	<50	<1.7	0
76	10047	<50	<1.7	<50	<1.7	0
77	10048	<50	<1.7	<50	<1.7	0
78	10049	<50	<1.7	<50	<1.7	0
79	10051	<50	<1.7	<50	<1.7	0
80	10054	<50	<1.7	<50	<1.7	0

Samples number	Patients ID number	Viral Load				Log different (bDNA -Real Time)
		Bayer® HIV-1 RNA 3.0 Assay (bDNA)		One-Step RT Real-Time PCR assay		
		(copies/ml)	Log	(copies/ml)	Log	
81	10056	<50	<1.7	<50	<1.7	0
82	10189	<50	<1.7	<50	<1.7	0
83	10190	<50	<1.7	<50	<1.7	0
84	10191	<50	<1.7	<50	<1.7	0
85	10192	<50	<1.7	<50	<1.7	0
86	10193	<50	<1.7	<50	<1.7	0
87	10194	<50	<1.7	<50	<1.7	0
88	10195	<50	<1.7	<50	<1.7	0
89	10196	<50	<1.7	<50	<1.7	0
90	10283	<50	<1.7	362	2.5	≤-0.8
91	10284	<50	<1.7	155	2.1	≤-0.4
92	10235	<50	<1.7	<50	<1.7	0
93	10303	<50	<1.7	<50	<1.7	0
94	10304	<50	<1.7	<50	<1.7	0
95	10306	<50	<1.7	<50	<1.7	0
96	10307	<50	<1.7	<50	<1.7	0
97	10308	<50	<1.7	<50	<1.7	0
98	10297	<50	<1.7	<50	<1.7	0
99	10296	<50	<1.7	<50	<1.7	0
100	10299	<50	<1.7	<50	<1.7	0
101	10301	<50	<1.7	<50	<1.7	0
102	10364	<50	<1.7	<50	<1.7	0
103	10365	<50	<1.7	<50	<1.7	0
104	10366	<50	<1.7	<50	<1.7	0
105	10367	<50	<1.7	<50	<1.7	0

Note: HIV-1 RNA viral load level <50 copies/ml (<1.7 $\log_{10}$) was used as 50 copies/ml (1.7 $\log_{10}$) for statistical calculation.

In additional, the correlation and mean difference between Bayer® HIV-1 RNA 3.0 Assay (bDNA) and in-house One-Step RT Real-Time PCR are shown in Table 12. The result was divided to 2 groups. For samples above 500 HIV-1 RNA copies/ml, the two assays were approximately 76% correlated, and the mean difference in $\log_{10}$ copy number between the two assays was 0.18 $\log_{10}$. For samples with 500 or fewer HIV-1 RNA copies/ml, the correlation between bDNA and in-house One-Step RT Real Time PCR results was less strong (approximately 11% correlated) and the mean difference in $\log_{10}$ copy number between the two assays was -0.10 $\log_{10}$.

As shown in Table 13, at the cut-off of HIV-1 RNA 50 copies/ml by Bayer® HIV-1 RNA 3.0 Assay (bDNA), the sensitivity and specificity of the in-house One-Step RT Real-Time PCR is 90.41% (66/69) and 90.62% (29/36), respectively.

Bland-Altman Analysis

In addition, the two assays were compared by using the Bland-Altman plot. The result is present in Figure 16. The bias is computed as the value determined by one method minus the value determined by the other method. The average of the differences will be close to zero. If it is not close to zero, this indicated that the two assay methods are producing different results. In this study, the limits of agreement defined as the mean difference in $\log_{10}$ HIV-1 copies/ml was 0.18 $\log_{10}$, ± 2 standard deviations (± 2SD) were -1.5 to 1.5. Bland-Altman plotting showed that differences between the $\log_{10}$ copies/ml results obtained from One-Step RT Real-Time PCR and Bayer® HIV-1 RNA 3.0 Assay (bDNA) were within (-1.75) – 1.37 log10 copies/ml of the averaged $\log_{10}$ results of the two tests for 95% of the specimens tested. This indicates that there is 0.187 $\log_{10}$ average discrepancy between both assays where the result from One-Step RT Real-Time PCR was 1.75 $\log_{10}$ lower and 1.37 $\log_{10}$ higher than Bayer® HIV-1 RNA 3.0 Assay (bDNA).

Table 12 The correlation between the results of log_{10}-trasnsformed quantification values obtained with Bayer® HIV-1 RNA 3.0 Assay (bDNA) and in house One-Step RT Real Time PCR

Assay range[a] (HIV-1RNA copies/ml)	N	Correlation (Pearson)	Mean log_{10} difference	95% Confidence Interval of the Difference	*p* value
<50-322,722	105	0.784	0.18	0.03-0.34	0.02
501-322,722	59	0.76	0.41	0.17-0.65	0.01
<50- 500	46	0.11	-0.10	(-0.25)-0.40	0.21

[a]Assay range was determined with Bayer® HIV-1 RNA 3.0 Assay (bDNA). CI: confidence interval.

Table 13 The sensitivity and specificity of in-house One-Step RT Real-Time PCR as compared to Bayer® HIV-1 RNA 3.0 Assay (bDNA) at the cut-off of HIV-1 RNA 50 copies/ml.

One-Step RT Real-Time PCR assay	bDNA assay[a]			sensitivity	specificity	accuracy
	Positive	Negative	Total			
Positive	66	3	69	90.41 %	90.625 %	90.47 %
Negative	7	29	36			
Total	73	32	105			

[a]Assay range was determined with the bDNA 3.0 assay.

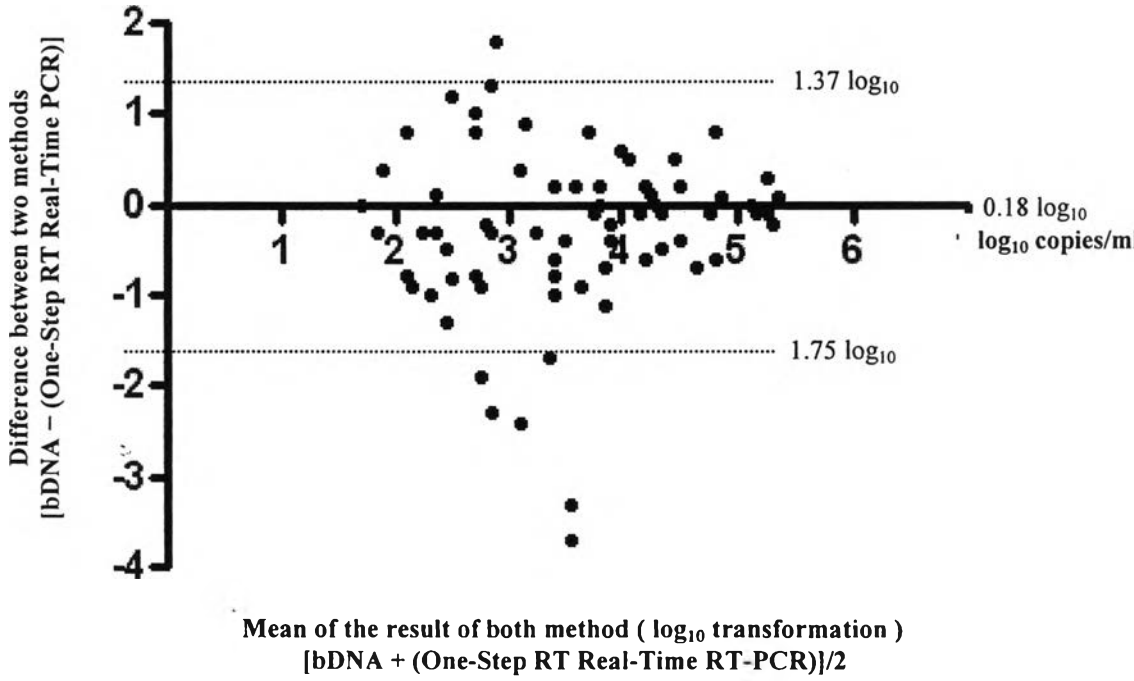


Figure 16 Bland Altman plot determined as Bayer® HIV-1 RNA 3.0 Assay (bDNA) and in-house One-Step RT Real-Time PCR

PART V. Overall cost comparison for Bayer® HIV-1 RNA 3.0 Assay (bDNA) in-house One-Step RT Real- Time PCR

The overall cost for Bayer® HIV-1 RNA 3.0 Assay (bDNA) and in-house One-Step Real-Time RT-PCR are shown in Table 14. The cost of the assay kits were determined according to the Baht price list (as of January 2005), although kit prices may be reduced according to discounted pricing differed by the manufacturer. Labor coat are noted in terms of time (minutes). The cost of disposables and labor for the two assays were compared for each of the three work flow steps: sample preparation, amplification/ hybridization/ detection and analysis. The in-house One-Step RT Real-Time PCR assay was performed in cost; labor and waste were less than Bayer® HIV-1 RNA 3.0 Assay (bDNA).

Table 14 Cost comparison between Bayer® HIV-1 RNA 3.0 Assay (bDNA) and One-Step RT Real-Time PCR

Cost category	Bayer® HIV-1 RNA 3.0 Assay (bDNA) (1run, 24 tests)		One-Step RT Real-Time PCR (1 run, 24 tests)	
	All tests (baht)	Per test (baht)	All tests (baht)	Per test (baht)
Assay components	31,248	1,302	5,784	241
Disposables				
Specimen preparation	600	25	4,080	170
Amplification /hybridization/detection	5,640	235	4,800	200
Analysis and report	24	1	24	1
Total steps	37,512	1,563	14,688	612
Labor				
Specimen preparation	970 min	970 min	300 min	30 min
Amplification /hybridization/detection	195 min	195 min	150 min	150 min
Analysis and report	30 min	10 min	30 min	10 min
Total steps	1,195 min	1,175 min	480 min	190 min
Biohazardous waste (total steps)	350 cm^3	14.5 cm^3	211.2 cm^3	2.2 cm^3