CHAPTER 5

DISCUSSION

In Thailand, an increasing of HIV-1 infected patients was continuously existed during the decade. Plasma virus quantitation is required for monitoring of antiviral drugs response and disease progression in these infected individuals. Based on the highly sensitive, specific and less carry-over contamination, real-time PCR technique is most widely used for quantitation of several pathogens including HIV-1. Nowadays, a number of commercial techniques for virus quantitation was established and huge of these tests were consumed in many epidemic regions, particularly in developing countries. Regarding to the resource limiting in several countries, an in-house real-time PCR assays for HIV-1 virus quantitation has been employed by many groups of researcher [39-45, 55]. An inexpensive and reliable real-time PCR for quantitation of HIV-1 RNA load from plasma samples was attempted to develop in this study. This validated technique was compared with reference commercial assay. Based on the highly conserved region, HIV-1 *gag* gene was selected as target for construction of control viral RNA in this study. HIV-1 pro-viral DNA gene was extracted and amplified by PCR. After PCR cloning, sequence integrity of HIV-1 *gag* gene inserted into the vector was analyzed by direct sequencing and aligned with the available sequences on GenBank database. The data indicated over 99% similarly with HIV-1 subtype CRF01_AE. The HIV-1 specific primers and probes were designed based on the conserved *gag* region of HIV-1. The amplified product was

110 bp. Approximately 60-150 bp was appropriated for real-time PCR validation [56]. HIV-1 specific probe sequence was replaced with scrambled sequence for internal system control (IC) construction by splice-overlapped extension PCR (SOE-PCR). This fragment was amplified and cloned into TA cloning vector. Internal system control RNA utilized from *in vitro* transcription was applied for detection of PCR inhibitors in individual plasma.

Avogadro's equation was applied for copy number determination of both synthetic HIV-1 RNA and IC-RNA. Optimization of IC-RNA was performed and an appropriate number used for individual tube of virus quantitation was 10^3 copies/ml. External standard curve used for viral load quantitation in plasma samples was generated by plotting of the various dilutions of synthetic HIV-1 RNA against cycle number over the cut-off threshold.

Assay limitation was evaluated using validated assay. Synthetic HIV-1 *gag* RNA was ten-fold serially diluted in normal human plasma and analyzed by real-time PCR. The result implied that the low level of virus was unable to detect by the established method since the linear range of virus detection appeared to be 10^3–10^{10} copies/ml. Although, the reference method is more suitable for low level virus quantitation ranging from 40-10^7 copies/ml, our developed method was superior in high level of virus quantitation with an approximately 3 log10 of virus detection [39, 40, 45, 55]. Assay reproducibility testing was performed by intra- and inter-run assays. The % CV was calculated and indicated a good reproducibility ranging from 0.99-2.18 for intra-run assay and 1.65-4.58 for inter-run assay, respectively. However, the

% CV was significantly increased while the low copy number of synthetic RNA was determined. According to the specificity determination, 40 sero-negative plasma samples were examined. Undetectable fluorescent signal was observed in all sero-negative samples indicating that our validate assay represented a highly specific. A total of 192 positive plasma samples with viral load ranged from less than 40 to approximate $1.7x10^6$ copies/ml were analyzed by our validated method. About fifty percent (93/192) of less than 1.0 log difference was accomplished using the validated test. Approximately 2.6% of samples (five samples with the plasma viral load of 431, $1.66x10^5$, $2.5x10^5$, $3.75x10^5$ and $5.13x10^6$ copies/ml detected by the kit) were defined to be contaminated with PCR inhibitor thereby presenting no signal in dual fluorescent in established method. According to the inhibitor detection, previous data revealed an approximately 3.7% which represented false negative results by PCR inhibitors [43]. About 70% of less than 1 log difference was observed in the samples with copy number more than 10^3 copies/ml. The plasma volume and long term plasma storage might be correlated with undetectable results in some of HIV-1 low copy number [44, 55]. An increasing of plasma volume has been previously reported to resolve this limitation [55, 57, 58]. To improve our limitation, 1,000 µl volumes of five positive plasma samples with the viral load about 100-300 copies/ml was tried out. Prior to extraction, plasma was high speed centrifuged at 23,600 g for one hour [60]. Eight hundred microliter of each sample was discarded and 200 µl was extracted according to manufacturer's instruction and further analyzed by using validated assay. Approximately 80% (4/5 samples) could be detected by our real-time PCR (Figure

22). Thus, the result implied that a larger plasma volume is required while the sample with low copy number of virus is determined. In addition, because fluorogenic system of TaqMan probe chemistry used in this study was depended on the 5'-exonuclease activity of DNA polymerase. The difference result in this study might be caused of the use of different DNA polymerase [59].

The correlation of both validated and standard method was performed by using Pearson's correlation coefficient (r) and Bland-Altman plot. After data analysis, the strength of linear association between the log values of validated method and reference method presented the high correlation with calculated R^2 of 0.9032 (with 10^3-$1.7x10^6$ copies/ml) and represented a good agreement in between both methods. The validated assay required only 200 µl plasma samples that suitable for use in adults and children [39]. Furthermore, the cost per test was about 560 Baht in which 4 times less expensive than standard method (varied from 2,000-4,000 Baht).

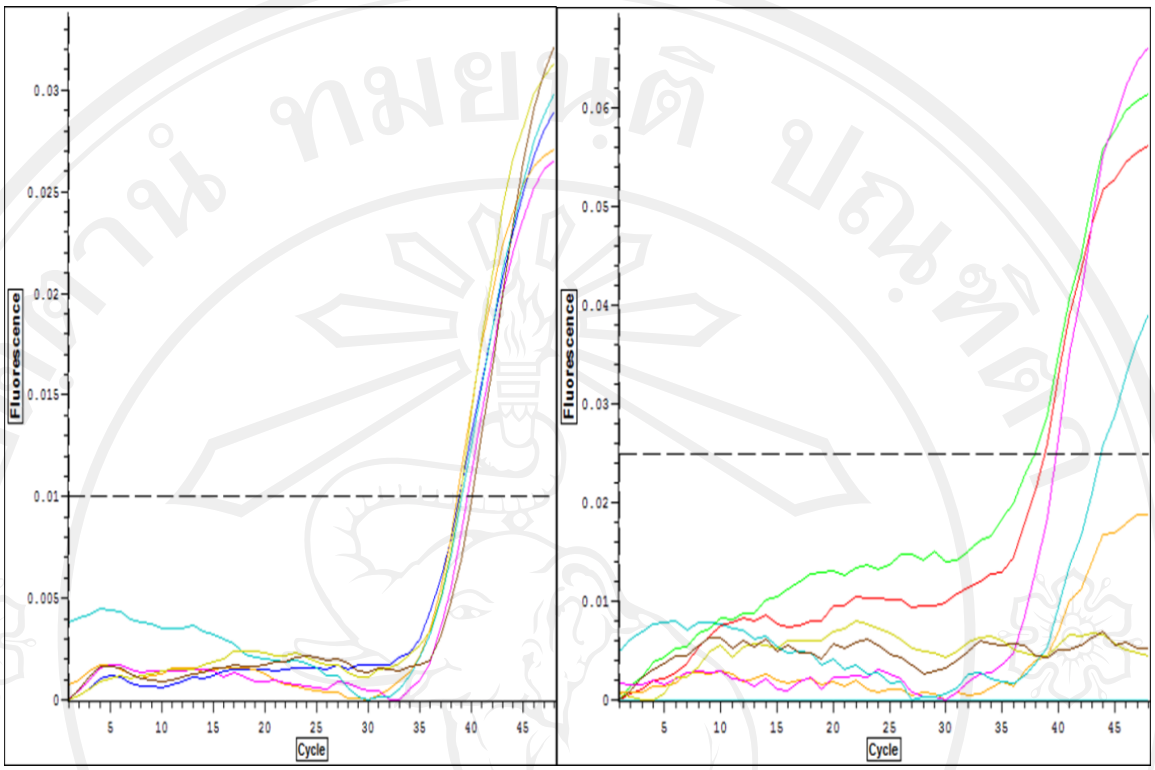

Figure 22 The fluorescent signaling curve of 5 positive plasma samples that was pre-concentrated by high speed centrifugation and re-analyzed by real-time PCR.

Hundreds copies per milliliter of five positive plasma samples that represent undetectable results by our real-time PCR, were re-examined. Approximately 80% (4/5 samples) could be detected by our real-time PCR when one milliliter of these samples were tested. 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.