CHAPTER V

DISCUSSION

Management of HIV-1 infected individuals has greatly successful with the introduction of highly active antiretroviral therapy (HAART). HAART is proposed to suppress HIV-1 replication as much as possible in order to attain durable virologic responses. The decrease of plasma viral load is closely linked with the improvement of clinical outcome and success of treatment (65, 66). With successful of HAART, the HIV-1 viral load level dropped below the limit of detection (<50 copies/ml) has been accepted in general (67-70). Thus, accurate determination of the viral load level became a basic tool for monitoring of HAART therapy and prognosis of disease progression, but the assays are available only commercially and too expensive to implement in a large-scale setting.

Scaling up of HAART due to a significant price reductions during last few years makes the urgent need of the low-cost HIV-1 monitoring assay especially in the resource-limited countries. One strategy is to develop an in-house assay which reliable, less expensive, high-throughput and easy to perform for large number of samples. Different in-house assay formats have been developed recently by many groups of investigators and have also been applied to quantify several kinds of virus pathogens (11, 43, 71).

Earlier quantitative assays were based mainly on conventional PCR methods (72). Those PCR methods (competitive or limiting dilution PCR) are based on end-point analysis and determine the amount of PCR products after the run is completed (the plateau phase). For the end-point method, a serious drawback is that reactions with different initial template number and/or different PCR efficiency can reach the same plateau. Thus, measurements taken during the plateau phase cannot easily differentiate variations in amount of starting material.

In contrast, the real-time PCR technology is a real-time analysis of PCR products which employ the fluorescent detection system to measure the amount of PCR products at each and every cycle and allows the course of a PCR to be visualized as a curve that contains an initial lag phase, an exponential (log-linear) phase, and a final plateau phase. The initial lag phase (background phase) lasts until the fluorescence signal from PCR product is greater than the background fluorescence of the probe system. The exponential log phase begins when sufficient product has accumulated to be detected above background and ends when the reaction enters the plateau phase. The real-time PCR provides accurate quantification because it allows data analysis only in the log-linear phase where the amplification efficiency of each reaction is constant and the initial template is varied directly to the signal detected (9).

Moreover, real-time PCR amplified and quantified target RNA simultaneously in sealed capillaries or plates that were never opened following amplification, minimized the risk of PCR product carry over contamination. Thus it did not need the dedicated laboratory space necessary for standard PCR.

The in-house assay developed in this study was the real-time PCR based assay using LightCycler™ instrument. This assay had offered several advantages.

First, the assay used HIV-1 positive pooled plasma collected locally to create the external standard curve. This homologous sequence can avoid the problem of differences in efficiency of amplification between unknown target RNA and the standard RNA. Since the pooled plasma was collected locally and within the same period of epidemic, the sequence variation between unknown and standard RNA was minimized. Although standard RNA can be obtained from *in vitro* transcription of linearized plasmid DNA carrying the cloned target sequence, but it required advanced technology that could not performed conveniently in the routine service laboratory. In contrast, the pooled plasma could be prepared in any laboratory with no additional expense but some disadvantage may due to the lack of plasma with high level of viral load after long term of HAART. However, we have shown in this study that the external standard curve created by using HIV-1 positive pooled plasma has a good performance with low error rate and well linearity.

Second, the assay sensitivity using 1 ml of plasma is comparable to the commercial test kit where the lower limit of detection was 100 vs.50 RNA copies/ml.

With this level of sensitivity, the assay could be used to monitor even in patient with successful HAART therapy. The assay was precise and reproducible with good intra- and inter run SD and CV and excellent correlations (r = 0.99) were obtained between in-house assay results and those obtained from Amplicor HIV-1 Monitor® test.

Third, the plasma viral load could be accurately quantified by using master standard curve. The highly correlation (r = 0.988) was observed between the result analyzed with master standard curve and the standard curve that run in parallel. Moreover, using master standard curve, the cost of assay may reduce about 10% for the maximum running (31 samples).

Fourth, the assay has low cost, less hand-on time and high-throughput. The assay cost of one plasma sample was about 500 Baht including the expense of external standard curve whereas only 450 Baht when master standard was used. No matter which types of standard curve were used, the cost of in-house assay was 5-7 times lower than the commercial tests. This in-house assay is therefore, suitable to use for monitoring the viral load in patients. With the LightCycler™ technology, 28-31 (maximum capacity including standard RNA) clinical samples could be processed and obtained the viral load result within less than 3 hours, contrast to most commercial test kits; Amplicor HIV-1 Monitor® test kit requires 6-7 hours, bDNA requires 4-6 hours. However, the capacity of assay is largely dependent on the types of machine used. The air-type chamber; LightCycler™ and Rotor Gene™ (Corbett Research, Australia) have maximum capacity at 32 samples whereas, the block type; Perkin Elmer/Applied Biosystem Division series (5700, 7000 and 7700)(USA), Chromo 4 from Biorad-MJ (USA) or Eppendorf™ (Germany) using 96 well-block platform, have maximum capacity of 96 samples which is more high-throughput than those air-type chamber. Moreover, the test is easy, the entire process of assay starting from RNA extraction and amplification can be carried by only one trained technician.

However, the disadvantage of using real-time PCR technology is the expensive equipment cost. Although, the price of real-time PCR machine decreases a lot in recent year, it is approximately between1-5 millions Baht depending on the functions, capacity and material used. This cost is still rather high and not suitable to implement in every laboratories. The strategy of setting core facility should be considered especially in those limited resource countries including Thailand. Finally,

the in-house real-time PCR based assay developed in this study could represent one other low-cost alternative HIV-1 viral load monitoring assay.