CHAPTER VI

DISCUSSION

The quantitative evaluation of HIV-1 viral load in plasma is a pivotal marker for the diagnosis and prognosis of HIV-1 infection. In addition, the viral load yields basis information for therapy monitoring allowing a concrete analysis of treatment failure caused by emerging resistance to a specific antiretroviral compound[4]. For these reasons, studies on molecular procedures to determine viral load quantitatively have increased in recent years. Approaches such as Bayer® HIV-1 RNA 3.0 Assay (bDNA) [Bayer Diagnostics], Amplicor HIV-1 Monitor (Roche Molecular Systems) and Q-NASBA (Organon Technica) are widely used in the clinical series. However, the cost of the tests still remains expensive (more than 2,000 bath. or 50 USD.). The adventage of real-time RT-PCR is that it offers a diagnostic system to quantify specific amplicons synthesis during PCR cycles,[12] and the cost of this One-Step RT Real-Time PCR is less expensive (i.e. approximately a half or more cost reduction).

The quantification of retrovirus by means of real-time RT-PCR has been applied successfully recently in the case of both HIV-1[14, 41, 85-91] and HIV-2[80, 81, 92, 93]. This has introduced a range of advantages which include (i) a larger dynamic range eliminating the need for serial assays to accommodate sample material with widely differing RNA levels, (ii) an ability to estimate sample concentration during exponential PCR stages, allowing RNA copy numbers to be quantified with greater accuracy and (iii) the elimination of post PCR processing reducing cross contamination risks and facilitating processing of higher sample number[94].

In this study, an in-house One-Step RT Real-Time PCR was developed in which a 111-neucleotide conserved region of the integrase of the HIV-1 genome was amplified. To detect the synthesized amplicons, TaqMan probe was added to the reaction which enabled real-time detection and monitoring of the amplification reactions. By using real-time monitoring, reaction and detection could occur simultaneously, omitting a time consuming detection step. Such a novel for HIV-1 RNA quantitation must have high specificity, sensitivity, reproducibility and accuracy. Moreover, the analysis of large numbers of samples requires a rapid and manageable protocol that minimizes as much as possible post

PCR manipulations. Due to the release of the quenching effect on the reporter, the fluorescence intensity of the reporter dye increases. The TaqMan assay offers a sensitive method to determine the presence or absence of specific sequences. Therefore, this technique is particularly useful in diagnostic applications, such as the screening of samples for the presence or incorporation of favorable traits and the detection of pathogens and diseases[60].

Our approach presented height specificity as 22 of 25 HIV-1 negative healthy donor samples failed to show any positive detection. 3 false-positive samples [that were negative in The Bayer® HIV-1 RNA 3.0 Assay (bDNA)] were repeated, 2 samples showed negative result but one of 25 (4%) sample still was positive (462 copies/ml). Difference in specificity between The Bayer® HIV-1 RNA 3.0 Assay (bDNA) compared to One-Step RT Real-Time PCR testing may associate with some errors such as sample contamination. Thus, HIV-1 negative healthy donors used in this study are unlikely to be in the window period from a previous exposure at baseline. However, the routine use of HIV RNA based assays in persons who are presented for postexposure prophylaxis is not recommended, because the potential harm of a false positive result outweighs the benefit of very early diagnosis. Receiving an HIV RNA test result that is likely to be a false-positive but requires follow-up testing for confirmation will be extremely stressful[95].

The efficacy of the assay was determined initially using serial dilutions of HIV-1 integrase RNA transcript standard. The PCR cycle at which the FAM fluorescent signal exceeded was taken as threshold cycle (C_t) value. The C_t value was determined in five independent assays of 10-fold dilutions of HIV-1 integrase RNA transcript standard prepared in DNase-RNase free water. The sensitivity of the assay was 80% at 50 copies/ml input and 100% at all higher 500 copies/ml input samples.

This in-house One-Step RT Real-Time PCR method for the quantification of HIV-1 RNA was highly reproducible. As evidence by ten replicate amplifications for each standard dilution were performed in each assay and were used to calculate the intra-assay coefficient of variation (CV). Also fluorescent threshold cycle (C_t) values obtained on the different days on which the assays were performed were used to calculate the inter-assay coefficient of variation (CV). The CVs were especially low, <5% for intra-assay coefficient of variation (CV) and <6% for the inter-assay coefficient of variation (CV). To analyze the

accuracy and the possibility of residual PCR inhibitor after extraction, 5 replicates of 5 different dilutions of HIV-1_{IIIB} viral particle which obtained 2 different runs assay were used to calculate the intra-inter assay coefficient of variation (CV). The % CV was especially low i.e., < 7% for both intra-inter assays. Other studies with real- time RT-PCR systems have reported similar results, indicating that our assay has a high reproducibility [14, 61, 80, 90].

In-house One-Step RT Real-Time PCR was compared with The Bayer® HIV-1 RNA 3.0 Assay (bDNA) reference standard technique for the quantitative determination of HIV-1 viral load. This comparison clearly demonstrated good correlation between the two methods (r = 0.787, p = 0.024). However, the One-Step RT Real-Time PCR detected a viral load in some samples that were undetectable with The Bayer® HIV-1 RNA 3.0 Assay (bDNA), 3 of the 32 HIV-1 seropositive patients plasma classified by The Bayer® HIV-1 RNA 3.0 Assay (bDNA) analysis as <50 copies/ml were detected by the One-Step RT Real-Time PCR. This the specificity is 90.625%. Vice versa, some samples positive in The Bayer® HIV-1 RNA 3.0 Assay (bDNA) but undetectable in the in-house One-Step RT Real-Time PCR assay. Thus One-Step RT Real-Time PCR assay detected in 66 out of 73 as sensitivity is 90.41%. The accuracy of the in house One-Step RT Real-Time PCR method is 90.47%. In spite of the same detection limit achieved by both methods, this discrepancy may be explained either by two different RNA extraction and purification approaches used for the two assays or by different algorithms employed by each assay to distinguish positive results from negative ones using a specific ratio of fluorescence between sample and background[96]. Overall, our assay has a high specificity, sensitivity and accuracy of approximately 90%.

To further compare our in house One-Step RT Real-Time PCR with The Bayer® HIV-1 RNA 3.0 Assay (bDNA), Bland-Altman analysis was used by plotting the difference between the $\log_{10}$ transformed data of both methods against their means. Such a Bland–Altman plot can be used if "we want to know by how much the new method is likely to differ from the old"[97]. If two assays generate similar measurements, the average of the differences will be close to zero; if they are not close to zero, this indicates that the two assay methods are producing different results. In our study, the mean difference in $\log_{10}$ HIV-1 copies/ml was 0.187 $\log_{10}$ ± 2 standard deviations (± 2SD) of -1.5 to 1.5. Bland-Altman plotting showed that differences between the $\log_{10}$ copies/ml results

obtained from One-Step Real-Time RT-PCR and The Bayer® HIV-1 RNA 3.0 Assay (bDNA) were within (-1.75) – 1.37 $\log_{10}$ copies/ml of the averaged $\log_{10}$ results of the two tests for 95% of the specimens tested, the so-called limits of agreement. 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 The Bayer® HIV-1 RNA 3.0 Assay (bDNA). Changes in plasma HIV-1 RNA levels of 0.5 $\log_{10}$ are thought to reflect biologically relevant changes in viral replication; thus, the limits of agreement between the methods exceed what is considered a biologically relevant change. However, if a limit of <0.5 log10 is used, seventy-two of 105 samples (68.5%) were concordant between the in-house One-Step RT Real-Time PCR and The Bayer® HIV-1 RNA 3.0 Assay (bDNA). Using a difference of < 1 $\log_{10,}$ 92 of 105 (87.6%) of the results were concordant. Overall, we conclude that the assays cannot be used interchangeably. Nolte *et al.*[55] reported a low concordance of results (58%) in their comparison of two commercial assays for quantitation of HIV-1 RNA in plasma (Amplicor 1.0 assay as compared the Bayer HIV-1 RNA 2.0 Assay). The observed discordance between the results of different assays has led to the recommendation that clinicians should use one method to monitor individual patients over time or reestablish a baseline value if the assay method is changed[98].

The assays for the routine monitoring of HIV-1 RNA levels in HIV-1 infected subjects should be reliable, reproducible, highly sensitive, practical, and cost-effective. The in-house One-Step Real-Time RT-PCR assay described here is simple to use and has been shown to be reliable both in reconstruction experiments and for the clinical use in the field. However there are rooms for improvement to this in-house development assay: Adding internal control and to further improve sensitivity. There was no internal control is added to the specimens prior to nucleic acid extraction as provided in part by commercially developed assays. Detection of viral nucleic acids requires a fully controlled RT-PCR workflow from sample preparation to analyze detection, so the co-amplification of an internal control should be added to exclude false-negative results. In a previous study, the successful method for quantitated HIV-1 RNA from plasma samples were co-extracted with Brome Mosaic virus (BMV) RNA as a internal control and amplified on HIV-1 gag region (position 684-903) by RT-PCR[99] and future improvement of this assay is to use the Brome Mosaic virus (BMV) as the internal control.

The One-Step Real-Time RT-PCR method was chosen because it requires less labor and sample handling, which reduces the risk of sample cross-contamination but one-step RT-PCR systems have been previously associated with poor sensitivity when compared with two step methods[100]. The problem of sensitivity will be most apparent with low copy RNA, primer-dimer formation, and the formation of non- specific products prior to the onset of the PCR phase of the reaction. To reach optimal sensitivity of the one-step assay, Hofmann-Lehmann R *et al.* reported not only reagent concentrations optimized, but also different enzymes for reverse transcription and amplification were evaluated[101]. In our study a combination of Mo-MuLV RT and Amplitaq Gold was used (TaqMan® One-Step RT-PCR Mater Mix reagent kit; PE Applied Biosystems). The *Taq* enzyme in both the PCR and RT-PCR master-mix systems is inactive until heated to 95 °C, after which the reactions are not cooled below 60 °C until the end of the analysis. In the case of the RT enzyme, there are no 'hot-start' enzymes available for this system and thus they have activity during the reaction set-up. Therefore, it is likely that the primers will anneal to one another and to non-target RNA during set-up and can thus form primer–dimers and non-specific products. Two step enzyme systems do not have the same problem as only a single primer is added to the mixture for the RT reaction. The forward primer is added only when the PCR reaction is carried out with 'hot start' *Taq*. One-step multiplex RT-PCR would be very difficult due to the requirement for multiple primer sets, which would lead to increase primer–dimer formation and loss of sensitivity. To resolve this problem a hot-start RT enzyme can be used to improve the sensitivity[102].

The other factor of loss of sensitivity is the yield of HIV-1 RNA from the RNA extraction step. Amplification of HIV-1 reverse transcriptase (RT) and protease (PT) sequences from plasma are difficult when HIV-1 RNA levels are low, and it usually cannot be accomplished in samples with <1,000 HIV RNA copies/ml. The RNA extraction step is critical for the success of subsequent amplifications and the efficiency of HIV RNA extraction methods is variable with low numbers of HIV RNA copies per milliliter[103]. We believe that our RNA extraction process results in higher loss of RNA than The Bayer® HIV-1 RNA 3.0 Assay (bDNA). In the latter assay, HIV-1 RNA was extracted based on magnetic beads and only one time transferred to a new tube. All of 120 μl HIV-1 RNA eluted solution was added directly to the microplate for amplification and analysis. For our RNA extraction protocol, 140 μl of one milliliter-centrifuged plasma sample was extracted based on QIAamp Viral RNA Mini kit and the HIV-1 RNA solution was transferred 4

times to new tubes. In the final step, HIV-1 RNA was eluted with 60 µl of distilled water and only 20 of this was added to the One-Step RT Real-Time PCR. We believe that HIV-1 RNA may be lost in all of these steps. To increase the sensitivity of One-Step RT Real-Time PCR the RNA extraction method could be altered. For example, The QIAamp® UltraSens™ Virus Kit (QIAGEN, Inc., Valencia, CA) could be used. In 2004, Michael Stuart Forman reported that his extraction protocol alterations by using the QIAamp UltraSens Virus Kit (QIAGEN, Inc., Valencia, CA), increased the clinical sensitivity of the assay since HCV was detected in patient specimens that were found to have no detectable HCV RNA after testing by the standard method. These specimens likely contained ≤ 50 IU/ml of HCV[104]. However, the cost of hot-start *Taq* enzyme and QIAamp® UltraSens™ Virus Kit are expensive, thus increasing the cost for in-house One-Step RT Real-Time PCR (1,300-1,500 baht).

Thus, to further improve this in-house One-Step RT Real-Time PCR the following approaches should be considered: 1) add an internal control 2) hot start RT-PCR reaction 3) improve the yield from the RNA extraction step. However, the down-side will be that the costs of our assay will increase.

In conclusion, our in-house One-Step RT Real-Time PCR assay has a good correlation, reproducibility, high sensitivity, and is practical and cost-effective. However, the agreement with the commercial The Bayer® HIV-1 RNA 3.0 Assay (bDNA) is poor and should be improved in the future prior to clinical implementation.