Development and clinical evaluation of an in-house human immunodeficiency virus-1 real-time RT-PCR quantitation assay

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NANYANG
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IN-HOUSE HUMAN IMMUNODEFICIENCY VIRUS-1
REAL-TIME RT-PCR QUANTITATION ASSAY**

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**SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING**

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ABSTRACT

Quantification of Human Immunodeficiency Virus (HIV-1) RNA plays a vital role in diagnosing and monitoring HIV virological behavior in patients. Currently, the HIV antiretroviral therapy aims to suppress the HIV replication to its minimal, thus quantifying HIV RNA is an indicative tool for assessing the therapeutics response. Despite its importance in HIV treatment, most patients have limited access to the viral load tests due to cost and resource constraints. Additionally, great variability among HIV genotypes and wide dynamic range of clinical HIV RNA levels has presented tough challenges for developing Polymerase Chain Reaction (PCR)-based amplification assays.

In this study, we describe the development, evaluation, and validation of a real-time RT-PCR assay designed to provide accurate quantification of viral RNA patients' specimens. This assay can perform a wide dynamic range of at least $8\log_{10}$ copies/mL. It has a high intra-assay precision with coefficient of variation (CV) of 0.825% and standard deviation (SD) of 0.04 $\log_{10}$ at $7.0\log_{10}$ copies/ μ L of external standards (ES); CV of 2.2% and SD of 0.23 $\log_{10}$ at $1.0\log_{10}$ copies/ μ L. It produces inter-assay precision of CV% of Ct value less than 4.2% and SD less than 0.9 PCR cycles for serially diluted ES of from 10 to 10^8 copies/reaction. The high precision and repeatability of this assay allow the derivation of a model for the viral load quantification result for determining antiretroviral drug therapy effectiveness. Probit analysis has determined the 95% confidence detection level is 61.5copies/ml. The genotype inclusivity of the Group M and N, combined with a small

sample volume requirement and low cost, makes this novel quantitative HIV real-time RT-PCR assay particularly well suited for application to high volume of clinical samples.

Clinical evaluation was also performed with samples from 329 HIV-1 positive patients in comparison with the commercially established assays: COBAS TaqMan HIV-1 and Abbott RealTime HIV-1 assays. Bland-Altman analysis showed good agreement with both assays; compared with COBAS TaqMan HIV-1 mean difference $-0.094\log_{10}$ and 94% of values within $1.0\log_{10}$; with Abbott RealTime HIV-1 mean difference $0.056\log_{10}$ and 99% of values within $1.0\log_{10}$. Frequency distribution was unimodal with similar modal peaks.

In conclusion, this HIV quantification assay developed is a reliable, easy to use, affordable assay that could increase access to reliable monitoring of response to therapy. The use of the ES control template from an in-vitro plasmid for quantification had also overcome the need of a high maintenance cost Biosafety Level 3 laboratory. The total cost of all the reagents was less than US\$10 per reaction. This assay had since been implemented into routine testing in Singapore hospital and research laboratories. It had been demonstrated to have the potential to be applied to a miniaturized point-of-care monitoring device.

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CHAPTER 1. INTRODUCTION

1.1 Background

HIV remains one of the most serious infectious disease public health challenges in the world today (UNAIDS, 2009a). Based on the update published by UNAIDS in 2009, people living with HIV has summed up to 33.4 (31.1-35.8 million) million with 2.5 million new infections and 2.1 million death in year 2008 alone (UNAIDS, 2009a). It is observed from the UNAIDS statistic that the number of people living with HIV was on an ever-increasing trend since 1990. With the beneficial effects of antiretroviral therapy and population's increased knowledge on HIV, the rate of increase in newly infected HIV cases have gradually slowed down. Despite recent success in treating HIV and increase access to life saving treatment that have increased survival and substantially decreased death rates, HIV remains a devastating illness, without cure, that mainly affect young adults. With its social and economic tolls as a threat of destabilize some developing countries, it continues to be a costly and controversial disease internationally. As a result, the WHO has ranked "combat HIV/ AIDS" as the 6th millennium development goal (United Nations Development Programme, 2000).

Figure 1: Global prevalence of HIV (1990 to 2008). Newly infected HIV cases and AIDS death (1990 to 2008) (UNAIDS, 2009a) .

Understanding the epidemiology of HIV is one of the important foundation for clinicians in recognizing risk behaviors associated with clinical manifestation suggestive of HIV infection in their patients, and encouraging acceptance of HIV testing, treatment to prevent opportunistic illnesses and delay disease progression. Although HIV and AIDS run across worldwide, some areas are more burdened than others. Sub-Saharan is one of the regions that are greatly affected with 1 out of 5 adults as HIV positive. With the United Nations' Declaration of Commitment on HIV/AIDS signed in 2001 and programs being executed, the number of newly-acquired HIV infections in sub-Saharan Africa has dropped by 15%, which was about 400 000 fewer infections in 2008. Similar

improvement is also witnessed in East Asia, where HIV incidence has also declined by nearly 25% and 10 % in South and South East Asia within the same time period. The epidemic is spreading most rapidly in Eastern Europe and Central Asia among drug injecting users, where the number of people living with HIV increased 67% between 2001 and 2008 (UNAIDS, 2009).

This global pandemic has been met by an unprecedented effort to bring available treatments (World Health Organization, 2008), especially in low and middle-income countries. In the 2006 UN political Declaration, which is led by countries worldwide with support from the UNAIDS, set the goals of a global commitment for scaling up the universal access to HIV treatment, prevention, care and support (UNAIDS, 2006). This plan has showed the success of the global commitment where AIDS death has finally showed signs of decrement in 2008. By December 2009 4 million people living with HIV in these countries were receiving anti-retroviral therapy, 31% of all who needed it. In order to maximize suppression of HIV virus and to stop the progression of HIV disease, a standard antiretroviral therapy (ART) that consisted of at least 3 antiretroviral (ARV) drugs would be used. As a result a potent ARV regimen, huge reductions have been seen in rates of death and suffering. While the HIV-1 positive patients are on treatment, they would be required to have routine viral load assessments to determine the effectiveness of drug therapy.

HIV-1 viral RNA quantification is the most sensitive prognostic marker of long-term response to treatment, with virological suppression to undetectable levels being the

primary endpoint for many randomized treatment trials and the key goal in many treatment guidelines. Virological suppression minimizes the risk of development of resistance on treatment. HIV-1 viral detection is the only means of diagnosing early mother-to-child transmission of virus. No surrogate clinical test currently available is able to take the place of viral load monitoring.

Access to viral load monitoring has been hampered by the cost of commercial assays. Some centers have developed in-house HIV quantification assays in an effort to improve throughput and lower cost. One of the potential limitations in this strategy is the need for Biological Safety Level (BSL) 3 laboratories for viral culture for internal standards. To overcome these issues, an affordable in-house viral quantification assay was developed, which has comparable performance as the current commercial kits with only the use of routine enhanced BSL 2 facilities.

1.2 Objectives

The objectives of this research project are:

1. To optimize the assay conditions of an In-House Human immunodeficiency-1 virus (HIV-1) real-time PCR quantification assay that is able to accurately and specifically quantify the HIV viral load from HIV patient blood plasma samples.
2. To evaluate the laboratory performance of in-house HIV-1 real-time PCR quantification assay using a HIV target control standard with statistical methods.

3. To evaluate the clinical performance of the In-House HIV-1 real-time PCR quantification assay against commercial quantification assays using 300 clinical samples.

1.3 Scopes of the project

This research will use a developed in-house detection kit for the use of quantification for HIV-1 subtypes that are commonly found in Singapore and Asia regions. It will also focus primarily on the validation of sensitivity, specificity and precision of the in-house detection and quantification of Human Immunodeficiency Virus Type-1 RNA in plasma using 329 patient samples provided by Tan Tock Seng Hospital. The in-house quantification will be compared with viral load results obtained by commercial assays currently used at our local hospitals.

CHAPTER 2. LITERATURE REVIEWS

2.1 Human Immunodeficiency Virus (HIV-1)

Human Immunodeficiency Virus (HIV-1) belongs to a group of retroviruses called lentiviruses, from the Latin *lentus*, meaning slow, because of the gradual course of disease they cause. HIV-1 viruses have diversified into different groups, namely groups M, N, O and P. The main HIV-1 group M (Major) viruses predominate and are responsible for the AIDS pandemic. Within group M, there is further diversified phylogenetic structure, where the HIV-1 strains are classified based on their genetic sequence data into different subtype. Till today, the subtypes that were found in Group M are subtypes A1, A2, B, C, D, F1, F2, G, H, J, K. Other groups present are the new N (non M, non O), P group and group O (outlier). HIV strains of Group O appeared to be restricted to neighboring countries in west-central Africa; group N strains were discovered in Cameroon in 1998. Till today, Group N remains extremely rare. In 2009 a new strain closely relating to gorilla simian immunodeficiency virus (SIV) was discovered in a Cameroonian woman. It was designated as the HIV-1 group P (Nau, 2009). Figure 2 shows phylogenetic analysis of the various groups and subtypes in HIV-1 virus.

Figure 2. Phylogenetic analysis of HIV-1 (Kuiken, 1999)

According to recent epidemiology studies performed on a global scale, the most prevalent HIV-1 genetic forms are subtypes A, B and C, with subtype B accounting for almost 50% of all HIV-1 infection worldwide. Subtype B is predominantly circulated across numerous continents such as the United States, Europe, Australia, Southeast Asia, northern parts of Africa and Russia. Subtype A viruses are predominant in central/east Africa regions (Kenya, Uganda, Tanzania, and Rwanda), and in east European countries such as Soviet Union. Subtype C viruses are found in southern Africa and India, where more than 80% of the global HIV-1 infected is populated. In addition, the relevance of CRFs in the global HIV-1 pandemic has gained its attention, with it accounting for 18% of incident infections (Hemelaar, Gouws, Ghys, & Osmanov, 2006). Recently, there have been surveillance reports on the local predominant form of CRF01-AE in Southeast Asia

and CRF02-AG in West and West-Central Africa (Menu et al., 1996, Motomura et al., 2000, Piyasirisilp et al., 2000). Figure 3 shows the epidemiology distribution of the various subtypes and CRFs prevalence of HIV infections worldwide recorded in the Los Alamos database, with subtype B accounting for almost 50% of the distribution. The surveillance of epidemiology distribution allowed the identification of the spread of the infection across borders. Thus, allowing the estimation of new introductions of new subtypes strain entering the countries for preparation of new drug treatment.

Figure 3. HIV-1 subtype epidemiology based on the available HIV genomic sequences on Los Alamos database (Los Alamos National Laboratory, 2010) .

2.1.1 HIV-1 virus and its genomics organization

Human immunodeficiency virus (HIV) is a lentivirus, a member of the retrovirus family. Figure 4 shows the schematic diagram of a HIV-1 virus with its structural and genetic contents namely the viral envelope, glycoprotein receptors, viral RNA and the critical enzymes for its replication. The HIV is spherical in shape with a diameter of 0.1 micrometer. When the new virus is formed in the host cell, it buds off by taking part of the host cell membrane for the formation of the viral envelope. Embedded throughout the viral envelope are complex protein structure known as Env, that spike protrude the envelope membrane. Env consists of a cap is made of three molecules called glycoprotein 120 (gp120), and a stem consisting of three molecules called glycoprotein 41 (gp41) that anchor the protein structure in the viral envelope. Inside the viral envelope contains a bullet-shaped nucleocapsid, made up of 2,000 copies of the viral protein, p24. The capsid encapsulates two single strands of HIV RNA, both encodes the complete genetic information of the virus. In addition, there are two important viral enzymes, integrase and reverse transcriptase that are essential for the propagation of HIV virus into the host system that is also inside the capsid.

Figure 4. Diagram of a HIV virion showing its vital structural and functional components (National Institute of Allergy and Infectious Diseases, 2009).

The HIV genomic coding RNA is a non-covalent dimer, with a 5' capped and 3' polyadenylated end, which annealed to a host tRNA^{Lys3} molecule (Coffin, Hughes, & Varmus, 1997). Viral proteins, especially nucleocapsid, chaperone the folding of HIV RNA. The integrated form of HIV-1, also known as the provirus, its RNA is approximately 9.8 kilobases in length. From Figure 5, it is observed that the genomic coding RNA contains nine open reading frames that encodes for 15 different proteins (Coffin et al., 1997; Frankel & Young, 1998) with both 3' and 5' ends of the genetic sequence flanked with repeated sequence known as the long terminal repeats (LTRs).

Figure 5. HIV-1 complete genomic map (Bette T Korber, 1998)

The HIV genome has several major genes coding for structural proteins that are found in all retroviruses that reflect its origin to retrovirus. However, it carries several nonstructural genes that are unique to HIV. These proteins are divided into major structural proteins: GAG, POL, ENV; regulatory proteins: TAT, REV; non-structural proteins: VPU, VPR, VIF, NEF. Each of these proteins plays different roles for the infection, proliferation and replication of the HIV virus in the host system.

After the 5'-LTR, the genome sequence presents the gag region. P55 myristoylated protein is the precursor of the Gag polypeptide that was then processed by the viral protease to form the functional proteins structures such as the matrix, capsid, nucleocapsid and p6 proteins. The 55-kDa Gag precursors play a role in viral assembly. Gag is associated with the outer coat of the virus plasma membrane, where virus assembly takes place (Scarlat & Carter, 2003). Furthermore, it is also involved in the maturation of the viron and early post entry steps in virus replication (Freed, 1998).

Following the Gag region is the Pol region, spanning 3kb long. The Pol genomic region encodes the critical viral enzymes such as the protease, reverse transcriptase, and integrase. The Gag-Pol precursor polypeptide is also processed by the viral protease to form the formation of these enzymes. Studies have shown that the Gag-Pol precursor is produced by ribosome frame shifting near the 3' end of gag (Freed, 1998; X Wu, 1997)

The next major structural protein-encoding region would be the Env, which spans 2800 bases long. From its name, the Env region encodes for the proteins found in the envelope of the HIV virus. The Env viral glycoprotein is first translated to as gp160, a precursor protein, before it is processed to form a non-covalent complex of the external glycoprotein (gp120) and the transmembrane glycoprotein (gp41). The mature gp120-gp41 proteins are attached by the non-covalent interactions. Gp120 possesses the binding site for the CD4 receptor, and together with 7 transmembrane-domain chemokine receptors, they serve as co-receptors for HIV-1. The binding of gp120 to host's CD4 cells activates subsequent functional interaction with co-receptor, which leads to membrane fusion of the HIV to host cell.

Other than the structural protein encoding genome, the HIV RNA genome also contains other encoding regions for regulatory proteins such as TAT, REV and VIF. These are the essential proteins for HIV gene expression. Trans-Activator of Transcription (TAT) is a transactivator for HIV gene expression and is localized primarily in the nucleus. The binding of TAT RNA element will activate the transcription initiation and elongation

from the LTR promoter, which would prevent the 5' LTR AATAAA poly-adenylation signal from causing premature termination of transcription and poly-adenylation. Regulator of Virion (REV) is a second necessary regulatory factor of the HIV gene expression. REV moves rapidly between the nucleus and the cytoplasm. When bound to RRE, it would promote the nuclear export, stabilization, and utilization of the viral mRNAs containing RRE. The regulatory function of REV is considered the most functionally conserved among the regulatory proteins of lentiviruses (Seelamgari et al., 2004; Yasmath F. Ahmed, 1990)

Another regulatory protein, viral infectivity factor (VIF) is a basic protein that promotes the infectivity of the virus, without inducing the production of viral particles. Without the presence of VIF, the produced viral particles are defective. However, this does not affect the cell-to-cell transmission of virus significantly. Found in almost all lentiviruses, VIF exists in both a soluble cytosolic form and a membrane-associated form; the latter is a peripheral membrane protein that is tightly associated with the cytoplasmic side of cellular membranes (Pomerantz, 2003; Strebel, 2003). Furthermore, it was discovered that VIF prevents the action of the cellular APOBEC-3G protein, which de-aminates DNA: RNA heteroduplexes in the cytoplasm (Mariani et al., 2003; Marin, Rose, Kozak, & Kabat, 2003; Yu et al., 2003). When the VIF is bound to the APBEC 3G protein, the virus would be signaled to degrade the VIF and as a result preventing it from infecting the host cell.

As for the remaining region, their functions remain under investigation. Various proposals have suggested that Vpr targets the nuclear import of pre-integration complexes, cell growth arrest, trans-activation of cellular genes, and induction of cellular differentiation. Vpu, on the other hand, is an integral membrane protein that was proposed to be involved in the degradation of CD4 in the endoplasmic reticulum, and enhancement of viron release from the plasma membrane of HIV-1-infected cells. Nef, a multifunctional 27-kd myristoylated protein identified in the nucleus and found to be associated with the cytoskeleton in some experiments. It was also proposed that Nef down regulates CD4, which enhances the signal transduction with the host cell and clathrin-dependent protein sorting pathways and therefore deduce to increase viral infectivity. Recent studies by Lake and Seelamgari have suggested that viral proteins, like Vif, have evolved to overcome the host's antiviral mechanisms, while proteins like Nef, which are markers for disease pathogenesis in vivo, seems to help increase pathogenesis by targeting bystander cells (Lake et al., 2003; Seelamgari et al., 2004).

Having the understanding of the replication mechanisms of the HIV virus and its infectivity mechanism allows researchers to yield new approaches for therapeutic strategies. By integrating information on the role of these regulatory and accessory proteins, their interactions with other viral and cellular components, and the subsequent effect on viral replication, the target gene region for the detection assay can be selected for this project. With the preference of selecting a well conserved region, and avoiding regions that play significant roles in the virus infection and replication, which are prone to mutation and drug targeted sites.

2.1.2 Diversity of HIV-1 genomics

HIV has very high genetic variability. This extensive diversity is a result of its fast turnover rate, with the generation of 10^9 to 10^{10} virions produced every day, coupled with a high mutation rate of the reverse transcriptase being about 3×10^{-5} per nucleotide base per cycle of replication. In addition, the highly recombinogenic nature of reverse transcriptase enzyme was well studied (Hu & Temin, 1990; Preston, Poiesz, & Loeb, 1988). This creates generations of different variants of HIV in an infected patient in a single day (Robertson, Hahn, & Sharp, 1995). This variability is compounded when more than 2 different strains of HIV infect the host cell simultaneously. With the co-infection, the genome of progeny virions may be composed of RNA strands from different strains. When this hybrid virion infects a new cell, the reverse transcriptase will replicate the 2 different strains randomly, generating a newly synthesized retroviral DNA sequence that is a recombinant between the two parental genomes. This therefore results in the formation of circulating recombinant forms (CRFs) derived from the recombination between viruses of different subtypes. CRF01_AE, for example, is a recombination between subtypes A and E.

2.2 Life cycle of Human Immunodeficiency Virus

HIV begins its life cycle upon binding its gp120 glycoproteins to the CD4 receptor of the host cell. CD4 receptors are found on the immune system's helper T lymphocytes the macrophages. Different strains of HIV enter the host cell with its particular combination

of receptor/co-receptor pairs. This enables the virus to fuse with the host cell by incorporating the lipid membrane of the HIV into the host cell's membrane as viral content enters the host cell. Figure 6 shows an illustration of the HIV life cycle in human.

Figure 6. Life cycle of Human Immunodeficiency Virus-1(AIDSinfo, 2005)

In addition to viral RNA, the virus carries enzymes that participate in the first few stages of the viral replication in the host cell. Both the HIV RNA and the enzymes will be

incorporated into the host cells after the fusion. During the replication process, the viral RNA reverse transcriptase copies the RNA into complementary DNA (cDNA). The viral RNA that has been incorporated into the cDNA is destroyed by another viral enzyme called ribonuclease H. Reverse transcriptase then synthesizes a second DNA strand with these cDNA being the template. The newly formed HIV cDNA then enters the host cell's nucleus, where a viral enzyme called integrase splices the viral DNA and integrates itself into the host cell's chromosomal DNA. The integrated viral DNA is known as provirus.

When the host cell is activated for replication, the provirus uses the host cell's DNA polymerase to create more copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA. These mRNA are used as a blue print to make long chain of HIV protein. The viral RNA encodes the capsid protein and other protein needed for viral assembly. Despite being in the spliced form, the RNA also encodes for the envelope protein and other auxiliary proteins. Envelope proteins are produced in the rough endoplasmic reticulum (ER) and then move through the Golgi apparatus before arriving at the cell surface. The HIV protease cuts the long chains of HIV proteins into smaller individual proteins. Full-length viral RNA molecules, along with other viral components, will then assemble at the membrane and bud off from the host cell. During budding, the new virus steals part of the host cell's outer envelope. This envelope, which acts as a covering, is studded with the HIV glycoprotein, gp120, which will be used to further infect the other host cells. These viruses would then be released into the host system and further infect other host cells as they circulate around the body (Scarlat &

Carter, 2003). During the process of virus invasion and replication, the HIV virus destroys cells of the immune system, making the body vulnerable to a host of diseases.

2.3 HIV-1 blood plasma viral load testing

HIV plasma viral load testing is used to obtain accurate quantitative measurements of HIV RNA in plasma. The quantification results are normally reported in copies per milliliter of blood plasma. The range of viral load is so wide that results are often given as results in terms of a logarithmic (log) scale. Log value is a measurement used to describe HIV and expresses the viral load values as a power of ten (written $\log_{10}$). The logarithmic scale was used because it was able to capture the large changes significantly on graphs or diagrams. This converted large numbers of copies/mL into ‘manageable’ figures. For instance, for most patients on treatment would have viral load of less than 50 copies/mL. This would then be reflected as $1.7 \log_{10}$ copies/mL. On the other extreme end of the scale, that would be frequently observed during the very early infection, a viral load of 10-million copies/mL, which would be equivalent to $7.0 \log_{10}$ copies/mL.

Measurements of HIV RNA viral copies in blood plasma are practiced routinely in clinical setting. Usually, patients will undergo HIV viral load testing for the initial evaluation of newly diagnosed HIV infection and for the surveillance of patients who are not receiving antiretroviral drug therapy. Before the clinicians begin any antiretroviral drug therapy or make any changes to the treatment regime, a viral load test will be ordered. This initial viral load reading would serve as the baseline Plasma Viral Load

(PVL) that is used as an important predictor of disease progression (Mellors et al., 1996). Researches have showed that HIV RNA plasma levels can be a powerful predictor of risk for disease progression. With the use of HIV RNA assays, the extraordinary rate of viral replication in vivo shortly after infection was observed. This debunked the earlier theories that the initial years of HIV infection were a relatively “benign” period. The significance of the viral load changes had served to trigger the need of early treatment to minimize the replication of the HIV virus in the host. The increasing problem of viral resistance in long-term management of HIV also becomes an obvious issue when the very high rates of viral reproduction were revealed (Piatak et al., 1993; Saag, 1997; Wei et al., 1995).

2.3.1 Importance of HIV Plasma RNA viral load testing

Clinical guidelines are available to guide physicians and patients through the complicated process of finding the optimal treatment regimen (World Health Organization, 2010). These guidelines are updated regularly to meet the needs of the ever-changing HIV situations worldwide with new discovery in the behavior of the virus and new drugs. The guideline includes regimens selections, when to initiate the regimens, recommended regimens for patients with co-infection of HIV and Tuberculosis and also solutions for treatment failures. Skillful selection of initial therapy is vital, as the failure of some medications can compromise the subsequent use of other antiretroviral drugs. Most of the drugs serve a common function, which is to minimize viral replication in the host. To determine if certain drug treatment is working, the use of viral load reading as a

measurement tool is highly recommended. Undetectable result using ultrasensitive assay, with detection limit of 50 copies /mL of blood is desirable.

Antiretroviral treatment in resource-limited settings has rapidly scaled up, however access to viral load monitoring continues to lag behind. The general misconception in the public seems to be that viral load testing is a tool that is great to have, but with drugs in hand, patients can make do without. However, this was proven otherwise where study has revealed how extensively a lack of viral load monitoring could undermine treatment. This study studied the frequency of HIV viral load testing that will help change the face of HIV patients. Based on a cohort of 2,333 patients across the Asia-Pacific regions, and it was found that patients were 35% more likely to develop severe HIV related illnesses, or die, when viral load monitoring was performed less than once a year. It was also shown that less than annual viral load testing reduced the odds of viral suppression. With the majority of the world's 33+ million HIV positive patients living in similar resource scarce settings, increasing viral load tests frequency could prevent up to millions of fatalities (Oyomopito et al., 2010).

Despite the importance of a viral load testing as a monitoring tool, it remains an uncommon practice in resource-limited settings. This is primarily because traditional test kits are expensive and demanding of both laboratories and the people running them. Therefore the need for development of inexpensive viral load test is in high demand.

2.3.2 Viral Load testing and its clinical implications

Recommended in the latest 2010 HIV treatment guideline, expansion in access of viral load test as a monitoring tool for treatment adherence and early detection of treatment failure is important for the success of second-line regimens (World Health Organization, 2010). Viral load tests can both inform the clinicians of the effectiveness of a treatment regimen and allows them to monitor adherence to the regimen – a frequent problem among HIV patients. The non-adherence to treatment often caused spikes in viral load readings. Furthermore, continuous monitoring provides vital information such as when certain drugs are no longer working and need to be altered. This phenomenon would be observed as a prolonged period of rising viral load reading as the HIV virus replication are no longer being suppressed. Viral load test serve as a multi-tasking tool to identify the treatment that is capable to more effectively suppress the virus and to prevent the development and passing on of resistant strains of HIV.

A number of studies have compared the prognostic values of plasma viral load (PVL) test with other traditional markers of risk for acquired immunodeficiency syndrome (AIDS). A study conducted in a large group of HIV-infected men found that PVL was the single best predictor of clinical outcome, the predictive values descend with the followed predictor measurement: CD4+ T-lymphocyte counts, neopterin levels, β_2 -microglobulin levels, and fever (Mellors et al., 1996). A similar study in HIV-infected women also demonstrated an association between PVL and disease prognosis (AM Rompalo, 1999).

Studies conducted by Hughes and O'Brien teams have concluded that the combination of PVL and CD4+ cell counts could better provide prognostic information than either factor alone (Hughes et al., 1997; O'Brien WA, 1997). Figure 7 shows the natural profile of human immunodeficiency virus (HIV) infection using plasma CD4+ T-lymphocyte counts and plasma HIV RNA levels. In most patients with HIV infection, initial viremia occurred within 4 to 11 days after exposure. When the virus has entered the host, it would replicate rapidly using the host cell before the immune system is triggered. Thus, RNA viral load would first peak before the rise in CD4 count is observed. As a result, RNA testing of HIV provides an earlier confirmation as compared to antigen testing which requires a waiting period of 2-3 months (commonly known as the window period) before accurate result can be determined. CD4 count and viral RNA count are inversely proportional during any point of infection. When the patient reaches the symptomatic stage or progressing to AIDS, CD4 count would drop drastically and HIV viral load reading would surge.

Figure 7. A generalized graph of the relationship between CD4 cell count and HIV plasma RNA count over the average progression of untreated HIV infection. (MA Sande, 1995)

These investigations also confirmed the ability of baseline PVL and CD4+ cell counts independently to predict clinical outcome and noted that after the initiation of antiretroviral drug therapy, any significant changes in these factors could predict outcome. Mellors' research team has showed that each 0.5-log₁₀ reduction in PVL is associated with a 30% reduction in the risk of clinical progression, whereas each 10% increase in CD4+ cell count has been associated with a 15% reduction in risk (Mellors et al., 1996). Moreover, in pregnant HIV-infected women, the PVL was able to predict the

risk of transmitting the virus to the fetus (Volberding, 1996).

Both the commonly used World Health Organization and International AIDS Society (IAS) HIV treatment guidelines for the management of HIV-infected patients have incorporated PVL testing for staging disease and determining prognosis. The IAS–USA Panel currently suggested that the results of PVL testing should be an essential parameter in decisions on initiating or changing antiretroviral drug therapy (Melanie A. Thompson et al., 2010). It was also recommended that measurements of PVL and CD4+ cell count should be performed periodically throughout the course of HIV treatment. (Volberding, 1996). Table 1 shows the PVL cut off and CD4+ cut off guideline from the USA panel during ART.

Table 1. Viral RNA and CD4+ count cut-off guideline for the use of antiretroviral agent in HIV-affected adults and adolescents. (Carpenter et al., 2000)

Anti-Retroviral therapy	RNA VL count copies/mL	CD4+ cell count
Start	> 30,000 copies per mL	< 350 per mm ³
Consider	5,000 to 30,000 copies per mL	350 to 500 per mm ³
Defer	< 5,000 copies per mL	> 500 per mm ³

2.3.3 Use of HIV RNA Viral Load in assessing the effectiveness of Antiretroviral Drug Therapy (ART)

Plasma viral load testing also has a role in optimizing antiretroviral drug therapy. Monitoring the viral load readings has the potential to improve clinical outcomes and avoid the use of antiviral agents that are no longer effective, thereby limiting the emergence of drug-resistant HIV strains. According to current International AIDS Society treatment recommendations, the preferred initial antiretroviral drug regimen is one that is most likely to reduce and maintain plasma HIV RNA below the level of detection (Gunthard et al., 2000; Kovacs et al., 1995).

The most important tools for evaluating treatment response are CD4+ cell counts and HIV RNA levels (Brambilla et al., 1999). Ideally, the HIV RNA level should decline rapidly after antiretroviral drug therapy has initiated. A 1- to 2-log reduction within four to eight weeks would be expected (Gunthard et al., 2000; Kovacs et al., 1995). When the target level of less than 50 copies per mL is not achieved after 16 to 24 weeks of treatment, clinicians would consider the possibility of drug resistance, inefficient drug absorption or poor compliance.

When a viral load below detectable level has been achieved for a considerably period of time, a general guideline is to change antiretroviral drug therapy if the plasma HIV RNA

concentration is found to be increasing. However, clinicians would usually delay changes of treatment until there is a documented consistent increment in the plasma HIV RNA level to greater than 2,000 to 5,000 copies/mL. In patients with an initially significant decrease in HIV RNA, (but not to below the detection level) and followed by a confirmed rebound to greater than 5,000 to 10,000 copies/mL might suggest the need for a treatment change. As this could reflect incompleteness of suppression of the virus replications and ineffectiveness of the drugs used (Gunthard et al., 2000).

Cautions are usually exercised when interpreting the results of PVL tests. Intra-assay and biologic variability might affect the findings, and concomitant illness or vaccination might cause transient HIV RNA elevations. In addition, all specimens are processed promptly (ideally, within 2 to 4 hours). Due to the rapid viral replication in vivo, patients who miss even a few doses of anti-retroviral drugs before their blood was drawn for viral load test may already be experiencing viral rebound, causing a surge in viral load. This “mirage” of viral load increment may lead to incorrect judgment of anti-retroviral drug therapy failure (Orendi et al., 1998).

Before ordering a PVL test, the physician would review the patient's adherence to the antiretroviral drug regimen and postpone testing if recent doses have been missed. Because lack of complete adherence to the regimen was often the primary reason for treatment failure, alteration of a failing regimen may not directly address the underlying

problem (Gunthard et al., 2000).

Although PVL testing is important, evaluating an antiretroviral drug regimen and making decisions on switching treatment should not be based solely on PVL fluctuations. Other factors such as declining CD4+ cell count, clinical disease progression, medications toxicity and intolerable side effects or the patient's non adherence should be taken into account before changing the drug regimens (Gunthard et al., 2000).

2.3.4 Viral load blip during viral RNA quantification

The current standard treatment for patients with HIV infection is highly active antiretroviral therapy (HAART). The goal of HAART is to minimize active viral replication so to avoid emergence of drug resistance by maintaining plasma virus levels below the limit of detection of current ultrasensitive assays (<50 copies/mL). However, many patients receiving HAART with full suppression of viral load to <50 viral copies/mL experience intermittent episodes of detectable low-level HIV-1 viremia >50 copies/mL, which are preceded by viremia in the undetectable range without any change in therapy. This phenomenon is known as blips. Studies have shown that up to 40% of patients under HAART experienced blips during the duration of their treatment (D Ward 2000; Gunthard et al., 1998; Havlir et al., 2001). However, persistent episodes of detectable or high copies of viral load are not considered blips (Havlir et al., 2001; Mira

JA, 2002; Raboud JM, 2002; Sklar PA, 2002). Such cases might be raise concern of incomplete suppression of viral replications or development of drug resistance. For patients with such situations, repeated measurement of the viral load would be required before the cause is established.

Two mechanisms have been well published to account for the blips of the plasma viral load during HAART. Firstly, activation of the latently infected memory pool during opportunistic infection such as tuberculosis and herpes simplex virus could result in the production of HIV particles, corresponding to the increase in plasma HIV RNA levels (Goletti et al., 1996; Orendi et al., 1998), after interleukin-2 administration (Kovacs et al., 1995), and after influenza or pneumococcal vaccination (Brichacek, Swindells, Janoff, Pirruccello, & Stevenson, 1996; Fuller et al., 1999; Gunthard et al., 2000; Staprans et al., 1995). Under such circumstances subsequent replication of HIV will be prevented by HAART and, consequently, no drug-resistant virus will be generated.

Secondly, blips of the plasma viral load during HAART could be caused by diminished suppression of HIV replication due to insufficient concentrations of antiretroviral drugs in the host system. This could be either a result of intermittent adherence to the regimen, temporary alterations in absorption of drugs, inhibitory interaction with other drugs, or ineffective entry at the sanctuary sites. As a result of the partial suppression of replication of the viruses, the replicating viruses may acquire drug resistance mutations (Martinez-Picado et al., 2000).

Using HIV blips as an early indicator of drug resistance development has been strongly debated among the HIV community. One camp of researchers like Cohen Stuart, Easterbrook, Macias, García-Gascó believed that blips in HIV RNA plasma load is associated with drug resistance (Cohen Stuart et al., 2001; Easterbrook et al., 2002; Garcia-Gasco et al., 2008; Macias et al., 2005). The researchers used an ultrasensitive genotyping assay to detect for drug resistance mutations before, during, and after blips. Among 2720 HIV patients on successful HAART during the 8-years study period, 779 (28.6%) developed at least one blip episode. Of which, 655 patients kept to their existing HAART regimen, following blip episodes were further monitored. After 12 weeks, undetectable viremia was reestablished in 458 patients (71%). These were considered to experience blips with neither drug mutations in the reverse transcriptase or protease genes detected at baseline. In contrast, 66 (9%) patients whose blip episodes were followed by virological failure, and drug resistance mutations detected in most cases, mainly in the retro-transcriptase or protease genes: rtM184V (66%) and rtK103N (29.5%).

For the other camp of researchers, they have attained data from recent studies to indicate that most blips are actually random biological and statistical variations around a mean viral load below detectable levels (<50 copies/mL) or due to false elevations of viral load from laboratory processing artifacts. Nettles's research team did a study to show that blips are representation of random variations around a mean viral load level <50 copies/mL (Nettles et al., 2005). Plasma viral loads were measured in two independent laboratories with the same ultrasensitive assay. Blips were detected in 9 of 10 patients and of the 713 total viral load measurements, 26 (3.6%) were >50 copies/mL, constituting

18 episodes of blips. Despite the use of the same assay with comparable sensitivities, the concordance between the viral load readings obtained by the 2 labs was poor. Moreover, the proportion of viral loads measurements >50 copies/mL was consistent with random biological and statistical variations around a mean viral load level of 10–20 copies/mL. In addition, most blips are not reproducible on duplicate viral load measurements (Nettles & Kieffer, 2006). Therefore, it appeared to represent a random biological and statistical variations rather than clinically significant elevations in viremia.

Blips might also be attributed to laboratory processing artifacts as evidenced by studies of which confirmed artificial elevations of viral loads with the use of Plasma Preparation Tubes (PPT) during collection of blood samples as compared with use of EDTA tubes (Stosor V, 2005). In the study, 56 patients who received HAART and with 3 consecutive undetectable viral loads from plasma collected in EDTA tubes, subsequently underwent an 8 month period of plasma collection with PPT. Significantly more patients (69.6% versus 5.4%, $P < 0.0001$) experienced blips with PPT used than with EDTA tubes. Among these patients, 60.7% of them had their blip episodes when the use of EDTA tubes was resumed. The manufacturers of PPT found similar results when comparing the two plasma collection methods and thus have issued guidelines for proper specimen processing when using PPT for the collection of blood for viral loads.

While blips generated by laboratory assay artifacts are of no direct clinical significance, they could significantly increase patient anxiety, complicate clinical decision-making and confound interpretation of research data. Given this, Nettles and Kieffer recommended

caution when using PPT for the collection of blood for viral load measurement, and urged thorough validations of new viral load assays and blood collection procedures when they are first introduced to for clinical purpose.

Therefore, blips in viral load is concluded not to be typically associated with the development of resistance mutations and most importantly are not associated with virological or clinical failure of previously adequate HAART. Laboratory variability could possibly result in fluctuation of viral load readings. Hence, taking into the account of statistical significance, minimal fluctuation in viral load within $0.5\log_{10}$ is the acceptable variability range in most of the clinical settings.

2.4 Current commercially available HIV-1 viral load kits

Various assays based on real time PCR detection technology have been developed for quantifying HIV-1 RNA in clinical samples. Of which, the two readily available commercial HIV-1 viral load kits, Abbott Real time HIV-1 (ART) (Abbott molecular Inc.) and COBAS Ampliprep/COBAS TaqMan HIV-1 test (Roche Molecular Systems, USA) were the most commonly used assay for HIV viral load testing worldwide and approved by the regulatory agencies in the USA(U.S Food and Drug Administration, 2011a).

Besides having the advantage of real time detection, these 2 assays have an initial step of automated nuclei acid isolation using magnetic beads. Studies have been shown that there is no significant difference when HIV RNA are extracted using automated or manual methods (Lesley E. Scotta, 2011). Therefore, in this research, they were used as comparator and evaluation reference assay tests. These two kits have been established as a standard quantification kit for the HIV viral load testing in Singapore hospitals. The ART and CTM test are both “locked-down” system where sample RNA extraction to the real time PCR to data analysis have been all integrated into a single system. Users of these systems have limited control on how the assay can be run and expensive reagents and consumables of these systems are only usable in their respective systems. These limitations have made the 2 commercial assays more costly than in-house assay, which are more open to other qPCR systems. Therefore, to ensure that the in-house HIV viral load assay could be a potential alternative or replacement of these commercial kits for the HIV patients in Singapore, the validation testing was performed and compared against them. Till date, there is no gold standard test for HIV-1 viral load testing (Daar et al., 2001; Owens, Holodniy, McDonald, Scott, & Sonnad, 1996). Most of the commercial viral load assays used in HIV viral load testing were established with high reproducibility. Therefore, as long as the same quantification assay has been used to assess the patient viral load consistently throughout the treatment-monitoring period, the viral load readings obtained at the different time points would remain as reliable monitoring data for the patients’ response towards the antiretroviral treatment.

2.4.1 Abbot RealTime HIV-1 assay

Abbott RealTime HIV-1 (ART) (Abbott Molecular Inc., Des Plaines, USA) is an in vitro reverse transcription-polymerase chain reaction (RT-PCR) assay for the quantification of HIV-1. This assay is accompanied with an Abbott m2000 automated sample preparation system, which used magnetic particle technology and ABBOTT mSample preparation kit for the plasma HIV RNA extraction. After the HIV-1 RNA has been extracted from 1mL of plasma, amplifications were performed on an Abbott m2000rt qPCR instrument. The internal control used in ART is derived from the hydroxypyruvate reductase gene from the pumpkin plant, encapsulated in an Armored RNA[®] particle, added to the lysis buffer prior to extraction. The use of this unrelated gene spiked at the beginning of the samples preparation step serves as a control to ensure the process has proceeded correctly in each clinical sample. The median amplification cycle of the internal control for each sample must be detected within an acceptable range, or else those samples have to be reprocess.

The ART assay kit consists of the ABBOTT RealTime HIV-1 calibrator kit, ABBOTT RealTime HIV-1 control kit and the ABBOTT RealTime HIV-1 amplification kit. The calibrator kit contains 2 calibrator reagents that would be used to generate the 6-points calibration curve for the quantitative determination of the HIV-1 in the clinical samples tested. The Abbott RealTime HIV-1 Calibrator A and Calibrator B contain armored RNA with HIV-1 sequences in negative human plasma. Calibrations are preformed with the both calibrators reagents run in triplicates. This calibration curve would be stored in the system and be valid for use for 6 months unless a new lot of samples preparation kit and

amplification kit is used. This limitation incurred higher cost for laboratories that have both low- and high- through put as the reagents packed in 24-tests are for single use only.

The control kit used in this assay consists of 3 controls: Negative control, Low Positive control and High Positive control. The 2 latter positive control contains Armored RNA with HIV-1 sequences in negative human plasma. These controls are used in every run to check for run validity. If negative or positive controls are out of range, all of the specimens and controls from that run must be reprocessed, from the beginning of sample preparation. The HIV-1 must not be detected in the negative control. Presence of HIV-1 detected in the negative control could indicate contamination across samples or by amplified product introduced during sample preparation or during preparation of the PCR.

The ART's amplification kit consists of a single reverse transcriptase and DNA polymerase enzyme, oligonucleotide 2 sets of primers and probes. One set of the primers is designed to target the integrase region of HIV-1, whereas the other set for amplifying the internal control. As there were 2 different pairs of primers amplifying 2 targets in the PCR reaction, any slight priming efficiency difference between the 2 primer sets would affect the amplification of the other targets. The probe complementary to the HIV target is labeled with a FAM fluorescence dye at the 5' end. A short oligonucleotide (quencher oligonucleotide) with a complementary sequence to the 5' end of the HIV-1 probe and is labeled with quencher molecule at its 3' end. In the absence of HIV-1 target, the HIV-1 probe fluorescence is quenched by FRET through hybridization to the quencher

oligonucleotide. In the presence of the HIV-1 target sequence, the HIV-1 probe preferentially hybridizes to the target sequence, dissociating from the quencher oligonucleotide, allowing fluorescent detection. The fluorescence signals accumulate with every cycle of the PCR and detected by the qPCR system.

The dynamic range of detection for the ART assay is $50\text{-}10^7$ copies/mL. It is able to detect HIV subtypes: Group M subtypes A—H, Group O and Group N, with 100% specificity (see Appendix C). The quantification of the control materials used in this assay has been standardized to the WHO standard.

2.4.2 COBAS Ampliprep/COBAS TaqMan HIV-1 test

COBAS Ampliprep/COBAS TaqMan HIV-1 test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) (CTM) is also a closed system starting from sample extraction till RNA detection. The automated isolation of the HIV-1 RNA on the COBAS Ampliprep instrument, using generic magnetic-silica based capture technique was followed with the real time PCR amplification and lastly data analysis with COBAS[®] TaqMan[®] Analyzer using AMPLILINK 3.1.1 software. The CTM assay consisted of a quantitative standard (known as HIV-1 QS), an armored RNA molecule that was added to the lysis buffer for every sample at the beginning of the extraction process. The HIV-1 QS construct contained the CTM's HIV-1 primer binding sequences and a unique probe-binding region. HIV-1 QS added to each specimen has a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification. This HIV-1

QS was used for the quantification of the HIV-1 RNA of the clinical sample, where it will take into account and compensate for the effects of inhibition and ensure that the sample preparation and amplification have proceeded correctly.

The CTM assay kit consisted of the sample preparation reagents and PCR amplification reagents. The sample preparation reagents (lysis reagent, protease, silica beads and HIV-1 QS) used for the RNA extraction were packed in reagent cassettes that can be easily loaded into the COBAS Ampliprep instrument. Both positive and negative controls are also included in the assay. Negative control is negative human plasma that was tested non-reactive by FDA licensed test for antibody for HCV, HIV-1/2, HIV p24 antigen and HBsAg and for nuclei acid materials from HIV-1, HCV and HBV. Two positive controls are included for the assay: Low- and high-positive controls, which are 100-times difference in their concentration of the armored HIV-1 RNA construct containing HIV-1 sequences. These controls are used in duplicates in every run to check for run validity. If negative or positive controls are out of range, all of the specimens and controls from that run must be reprocessed, from the beginning of sample preparation. The HIV-1 must not be detected in the negative control. Presence of HIV-1 detected in the negative control could indicate contamination across samples or by amplified product introduced during sample preparation or during preparation of the PCR.

As for the PCR reagents, it consisted of a master mix containing DNA polymerase, buffers, 1 set of primers and 2 TaqMan probes specific to the HIV-1 target and the HIV-1 QS for the amplification of the targets. The CTM's primers targeted the gag-region of

HIV-1, which would amplify the HIV-1 target and HIV-1 QS RNA. This was then followed by fluorescence detection by the cleavage of the dual labeled oligonucleotide probes that are HIV-specific and QS-specific respectively. Unlike the ART assay which used the calibration curve for quantification of the HIV-1 RNA, a lot-specific calibration constants provided with every lot of CTM test are used to calculate the viral load for the specimens and controls based upon the HIV-1 RNA and HIV-1 QS RNA Ct values. Instead of a calibration run performed before every new lot of test reagent being used, the CTM assay performed pre-check on the positive and negative control after every PCR amplification run before fluorescence readings of the clinical samples are processed to generate the quantification values. The dynamic range of CTM assay is $40\text{--}10^7$ copies/mL. It is able to detect HIV subtypes: Group M subtypes A—G. The quantification of the control materials used in this assay has been standardized to the WHO standard (see Appendix D).

2.5 FDA Guidelines for Development and Clinical Evaluation of in-vitro HIV test

2.5.1 FDA guidelines for HIV-1 Assay development

Under the U.S Food and Drug Administration (FDA) guideline, analyte specific reagents (ASRs) are defined to be "antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a

diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens." (U.S Food and Drug Administration, 2011b). Primer and probe oligonucleotides used in this in-house assay developed would be considered under the category of analyte specific reagents of FDA regulations. According to the guidance release by FDA for industries manufacturing nuclei acid tests for HIV-1, issues pertaining from the assay design and rationale to end-users storage and instruments have to be addressed to ensure assay performance. A detailed description of all aspects of the technique including sample preparation, assay optimization, amplification, and detection methods should be provided. Validated quality control procedures that are state-of the-art should be used to assure manufacturing consistency (U.S Food and Drug Administration, 1999). The details of each component will be discussed in this chapter, and as for the methods used in this research project to fulfill to the FDA regulations would be discussed in the next chapter.

During the initial stage of assay designing, design rationale of the detection assay such as the selection process of the target sequences that were used as template for the design of the primer and probe sequences need to be address. For the case of HIV-1, the subtypes and the number of sequences per subtypes that were used have to be stated. Having the template, the selection process of primer and probes sequences has to be attended to, including showing the degree of conservation of nuclei acid sequences. Since the HIV viral load assay is a quantitative assay, which requires quantification standards, the design and nature of these standards would therefore be considered.

The next phase after the in-situ designing comes the assay optimization. The optimization process includes determining the most favorable conditions to obtain excellent extraction yield from the sample, template amplification conditions and best efficiency for accurate quantification of the HIV-1 virus. During this phase of assay optimization, the optimal assay conditions, reaction kinetics and the lower bounds of reliable assay performance needs to be defined. For quantitative assays, the lower limit of reliable assay performance may be defined by two potentially distinct values; the lower limit of detection (LOD) and the lower limit of quantitation (LOQ). According to the FDA guideline, LOD is defined as the lowest concentration of HIV-1 RNA that can be distinguished from a negative specimen with a predefined level of assay sensitivity. On the other hand, the LOQ is the lowest concentration of HIV-1 RNA that is distinguishable from a negative specimen with the same degree of sensitivity as the LOD that is also quantifiable with an acceptable degree of precision and accuracy (e.g., CV of $\leq 35\%$). The type of internal control or calibrators used should also be clearly defined, with the LOD of these control be validated by other established characterization method or direct measurement such as UV optical density. Furthermore, the linearity covering the clinical utility range and the reproducibility and accuracy of the controls across the standard curve should be examined. Assay needs to be validated across multiple viral subtypes, with internationally accepted RNA standards. The performance of the LOD, LOQ, linearity, specificity, accuracy and precision of this in-house assay would be discussed in the result chapter.

The sample specimen used for the assay such as cells, whole blood, plasma or dry blood spots should be specific with the preparation steps necessary to ensure extraction yield is optimized. Controls should be used to monitor ultracentrifuge, extraction, amplification, quantification steps. Caution steps used to avoid contaminations and inhibition that would possibly be carried over during each step should be stated. The control used for monitoring purpose should be similar the specimen type whenever possible, with spiked control being co-amplified by the primers are acceptable. The steps used to verify these matters are discussed in the next chapter under sample preparation.

2.5.2 FDA guidelines for Clinical Evaluations

For the phases of clinical diagnostic assay development, clinical evaluation would be performed after the assay has been optimized in a laboratory. This usually performed prior to the assay being implemented for routine clinical use. Clinical trials are research studies that test how well new medical approaches work in people. The preclinical studies performed in-house or at field sites, are capable to provide preliminary information on the assay performance, such as clinical specificity, sensitivity and reproducible. Specificity and sensitivity study is a statically determination of the false positive and false negative. As recommendation by the FDA guideline, a minimum of 100 serologically positive samples should be tested to establish the specificity. On the other hand, a minimum of 300 patient samples should be tested the sensitivity assay. All the patient samples would also be required to run in parallel with appropriate competitor assay. For the development of this in-house viral load assay, the clinical validation study was performed with the

comparison of the in-house HIV viral load assay against 2 other commercially- available tests Abbott Real time HIV-1 (ART) (Abbott molecular Inc.) and COBAS Ampliprep/COBAS TaqMan HIV-1 test (Roche Molecular Systems, USA). A total of 329 patient samples were used in the clinical evaluation, among those, 151 HIV sero-positive samples were evaluated with COBAS test kits and 178 HIV sero-positive samples were evaluated with Abbott test kits.

Analytical sensitivity of the assay developed is also required by the FDA guideline to be performed by testing a serial dilution of at least 10 HIV positive samples that were evaluated with the appropriate tests that were current at the point of assay development. It is also recommended to run the samples against recognized reference panel (e.g., reference HIV panel produced by the WHO, CBER or the ACTG VQA). These recognized panel would be able to provide their viral loads in quantitative values in the internationally recognized unit. For a quantitative assay, the dilution series of these patient samples should cover the complete quantitative range of the assay under development. The LOD of the highest dilution should be reproducibly and consistently detected and therefore being defined in copies per unit sampling. For this assay, an external standard was synthesized from HIV-1 patient sample through cloning technique for the assay analytical evaluation. This external standard was calibrated against the WHO HIV reference panel. The usage of this abundant external standard has helped to enable reproducibility and precision test to be performed in multiple occasions. This external standard was then used as the quantitative standards curves in subsequent

clinical validations, which will be impossible to use the serially diluted patient samples due to their limited supplies.

In Singapore, the need for usage of human subject samples for the research studies would require the ethical board approval. This independent body constituted of medical/scientific and non-scientific members from all National Health Group (NHG) institutions as well as independent members from outside NHG formed the NHG's Domain-Specific Review Boards (DSRBs). DSRB's primary objective is to safeguard the rights, safety, and uphold the core ethical core principle of respect of human research subjects in the National Healthcare Group and her institutions. This is to ensure that the risks are small and are worth the potential benefits. All the above are ensure through proper documentation of the study protocol and study plan. The plan describes what will be done in the study, how it will be conducted, and why each part of the study is necessary. Continual reviewing, approving, and providing continuing review of research studies, of protocols and amendments, and of the methods and materials to be used in obtaining and documenting informed consent of the research subjects (National Healthcare Group, 2009). Approval from the DSRB has been obtained before the clinical cohort is recruited for providing blood samples for this comparison study of the in-house assay and the commercially available assay.

2.6 Literature review summary

In summary, HIV is a devastating disease that affects both socially and economically.

Being a populating disease without a cure, HIV treatment and its monitoring tools remain the key to control and delay this disease's progression into AIDS. Having understood the diverse and highly mutational behavior of the HIV virus, the need of having current and clinically functional HIV antiviral drugs and diagnostic kits is critical. The noteworthiness for research in these areas has gained strong support from the World Health Organization (WHO). HIV-1 RNA quantification is the most sensitive prognostic marker for long-term response to anti-viral treatments. However, access to viral load monitoring is impeded by cost of commercial assay. Commercially available assay are usually "lock-down" system where bulky equipment and costly consumables are tied together with the detection assay. As a result, users have limited control over the assay run when every procedure is locked up and automated. The stringent regulations set by the US FDA for commercially developed diagnostics kits has made it difficult for commercial kits to lower their cost and also to constantly update their assay with the emergence of new strains of HIV-1. As a result, in house developed diagnostics kits, which are regulated with a less stringent set of rules, have mushroomed in hospital laboratories to serve as a more affordable disease-monitoring tool for the HIV patients.

CHAPTER 3. MATERIALS AND MATHODS

3.1 Samples and materials

3.1.1 Sample source and extraction of viral RNA from clinical specimens

The ethics committee of Singapore National Healthcare Group has approved the use of clinical samples for this research study (DSRB reference number: B/07/457). HIV-1 infected patients (both treated and newly-diagnosed) at the Communicable Disease Center, Singapore scheduled for HIV-1 viral load testing or referred by their physicians from December 2007 to March 2009 were consented before sample collection. A total of 329 samples collected were assayed using the in-house assay. These samples were blinded to the operator for the purpose of patient confidentiality and integrity of the result for the research project. Two tubes of whole blood (3 milliliters each) were collected in the EDTA-blood tubes from each patient. One tube was sent to collaborator's laboratory for commercial HIV-1 quantification kits testing, while the other for the evaluation of this in-house developed assay. HIV-1 viral load was monitored by Abbott Real time HIV-1 test (ART) and COBAS TaqMan HIV-1 test (CTM) carried out by the staff of Tan Tock Seng Hospital, Microbiology Laboratory and National University Hospital, Molecular Diagnostic Center respectively based on manufacturers' protocols.

Whole blood samples collected and sent to the Tan Tock Seng Hospital, Infectious Disease Research Laboratory for sample processing. Presence of EDTA in the blood collection tubes prevented the coagulation of blood before the samples processing and allowed plasma to be collected for viral load testing. The whole blood was centrifuged at 15,000x gravitational field (xg) for 15 minutes within 6 hours time frame of collection time point. Centrifugation separated the plasma from the red blood cells (see Figure 8). Caution was practiced when liquating the top layer of plasma from the blood tube to the 2 mL cryovials and stored in a -80°C freezer in until sufficient samples are collected for the batching process of extraction.

Figure 8. HIV patient blood sample collected in EDTA blood tube before and after plasma separation via centrifuging at 15,000-x g for 15 minutes.

Sample extractions were performed in batches of maximum 24 samples due to the limitation in the refrigerated high-speed centrifuge available in the laboratory. In addition, to minimize the inter-batch variations and errors among the entire patient samples cohort studied in this research work, batch size has been maximized to the capability of all the equipment required in the process.

Prior to the extraction process frozen plasma samples were thawed and centrifuged at an ultra-speed of 24,000xg for 60 minutes at 4°C. Ultra-centrifuging served as a concentrating method to accumulate all the free viral particles in the plasma at the bottom of the micro-tubes (see Figure 9). 800µL of supernatant was then discarded gently with a micropipette without disturbing the pellet of viral clump, and the remaining 200µL of plasma containing the viral pellet was used for RNA extraction using the QIAamp Viral RNA Mini Kit (Cat #: 52906, Qiagen, Germany), following the manufacturer's protocol. Purified RNA was eluted in 50µL of 1x Tris-EDTA (TE) buffer and stored at -80°C. TE buffer used as storage buffer as it contained EDTA which is a chelating agent that take up all the divalent metal ions such as Mg^{2+} ions which are required by RNase and DNase for their activity. Furthermore, the buffering capacity of TE buffer helped to maintain pH. This helped to prevent the degradation of the purified RNA for longer storage period (Elmer M. Cranton, 2001).

Figure 9. Concentrating of HIV free viral particle (in red circle) via ultra-speed centrifuging at 24,000 xg for 60 minutes at 4°C prior to RNA extraction process.

3.2 Development of In-House HIV Viral Load assay

3.2.1 Oligonucleotide design

In-house primers and probes were designed using alignment data referenced from the Los Alamos National Laboratory database that was made publically available in 2008 (Los Alamos National Laboratory, Los Alamos, N.M). Total 163 sequences were selected from the database. Diversity of geographic locations and sampling times for each subtype, with an emphasis on more recent samples were reflected in this database alignment. More sequences from the more prevalent subtypes, only one representative for

each CRF (except for the widespread CRFs 01 and 02), and group N, group O, and CPZ sequences were included in multiple alignment. According the HIV Los Alamos National Laboratory database, its selection was intended to show the worldwide variation of HIV-1 subtypes and CRFs.

The *gag* gene from the HIV genome was chosen, as it is relatively well conserved in HIV-1 virus (Nakamura Y, 1997; Ogg, 1998). As seen in Figure 10, multiple sequences alignment of the HIV sequences from the HIV-1 2008 compendium was performed and a well-conserved region in *gag* was selected for the detection primers and probe. One set of primers sequences, designated gag183F/R (forward- 5'-CTAGCAGTGGCGCCCGAACAG-3'; reverse- 5'-CCATCTCTCTCCTTCTAGCCTCCGCTAGTCA-3') and a detection oligonucleotide TaqMan probe with a reporter fluorescence dye (FAM, 6-carboxyfluorescein) attached to the 5' end and BHQ-1 quencher linked to the 3' end as (FAM) 5'-TCTCTCGACGCAGGACTCGGCTTGCTG-3'(BHQ-1) was designed under the guidance and assistance of our collaborator, Dr. Masafumi Inoue from the Experimental Therapeutics Center (A*STAR). Both primers and probes were selected and analyzed using primer design software (OLIGO PRIMER ANALYSIS SOFTWARE, Molecular Biology Insights, Inc, Cascade, CO, USA). Maximum homology of the primers and probes with the various HIV-1 subtypes was ensured, without the formation of secondary or hairpin structure. The forward and reverse primers would amplify the HIV target based on complimentary base pairing, and produced a PCR product with a length of 183 base pairs, which contained the complimentary binding site of the TaqMan probe. The

selection of the primers was made based on the criteria of specificity and efficiency. During the selection of the primers, there was a need to ensure high specificity at the cost of some efficiency. It was a higher priority to avoid false positive as compared to producing large quantities of amplified product.

Figure 10. Multiple alignment of HIV-1 subtypes in the gag region. Conserved regions (seen as dotted regions in the alignment) are selected for the detection primer design.

3.2.2 External standard for real time quantification

External standard (ES) for the quantification of the viral RNA copies is required in the viral load assay. These standards were nucleic acid material of known quantitative concentration, in terms of copies/ μ L, that would serve as a quantification ruler for the unknown patient samples. A positive patient sample of subtype AE was used as the initial material for the synthesis of the external standard. The in-house primers were used to amplify the target region of the HIV genome for the quantification system. RT-PCR amplification of HIV-1 positive patient sample (subtype AE) was performed. To ensure a pure and exact external standard template is generated, two rounds of amplification and purification were performed. In the first round of RT-PCR, amplicons generated by the gag183U/L primers were verified by conventional gel electrophoresis and purified from 3% Agarose gel using QIAquick Gel Extraction Kit (Cat #: 28704, Qiagen, Germany), following manufacturer's protocol.

Next, the gel-purified product was further amplified using HotStarTaq Master mix kit (Cat #: 203443, Qiagen, Germany) using the same detection primers gag183U/L. Amplicons were purified using QIAquick PCR Purification Kit (Cat #: 28104, Qiagen, Germany) before cloning it with TOPO® TA Cloning® Kit for Sequencing (pCR® 4 TOPO® Vector) (cat #: K457540, Invitrogen, Carlsbad), following manufacturer's protocol. Plasmids were extracted and purified, before being serially diluted and stored in -30°C for future assay. The insert was confirmed by sequencing performed on an Applied Biosystem 3730xl DNA analyzer using Big Dye® Terminator (version 3.1) Cycle-

Sequencing Kit (Cat #: 4337455, Applied Biosystem, USA). The diluent reagent used to perform the standard dilution contained 0.1ng/μL of salmon sperm DNA (ssDNA) in 0.1xTE buffer. The ssDNA served as a carrier RNA to minimize the degradation of the purified plasmid. In the presence of RNase, DNase, ssDNA in the storage reagent served as a sacrificial role to be digested prior to the targeted samples. In addition, the short segment of ssDNA would not to interfere with the assay detection system and remains inert during the PCR assay. Figure 11 shows a flowchart of events that were done to obtain the nuclei acid of the ES.

Figure 11. Work flow of the synthesis of external standards for the In-House HIV-1 viral load quantification assay

The ES was calibrated against imported NIBSC HIV-1 RNA 2nd International Standard (National Institute of Biological Standards and Control, Potters Bar, United Kingdom).

Extracted NIBSC HIV-1 RNA standard was serially diluted and ran parallel against the ES. After the calibration was performed, it was found that the ES has a skewed quantification valued as compared to the international standards. Hence, to fulfill the purpose of ensuring the quantification assay developed for this research project was able to provide viral load reading on par with the international quantification units of measurement used worldwide, an adjustment factor of 2.49 (see Appendix A) was applied to the viral load readings obtained in this assay.

3.2.3 Internal control (IC)

Competitive internal control (IC) molecule represented an in vitro transcribed oligonucleotide molecule that was spiked to each RT-PCR reaction. . The purpose of having IC inside each of the reaction wells was to check for PCR inhibition. This oligonucleotide sequence of internal control was randomly generated with no homology with the human genomes. It consisted of regions where it allowed co-amplification by the primers gag183U/L. The internal control had a unique probe-binding site, which was different from that of the binding site of the HIV-1 target molecule incorporated. This single stranded oligonucleotide (5'GTGGCGCCCGAACAGTATCGCGTTTATGCGAGGTCGGGTGGGCGGGTCGT TAGTTTCGTTTTGGGTGCATAGCGGAGGCT-3') was synthesized by 1st Base Oligo synthesis facility. A detection oligonucleotide TaqMan probe with a reporter fluorescence dye (Texas Red) attached to the 5' end and BHQ-1 quencher linked to the 3' end as

(Texas Red) 5'-AGGTCGGGTGGGCGGGTCGTTA-3'(BHQ1), was designed to pick up the amplified molecules of the internal control in the PCR reaction.

To increase the integrity of the internal control molecule during the storage in the assay kits, the single stranded template was transcribed into double-stranded cDNA. PCR amplification was carried out using FINNZYMES Phire™ Hot Star Taq DNA polymerase (catalog no.: F-120S, Finnzymes, Finland) to generate the double stranded internal control. Internal control molecule was diluted using 0.1ng/μL ssDNA where ssDNA served as the carrier and sacrificial molecules in conserving the internal control molecules.

In a competitive PCR reaction, amplification primers had the preference to bind to their complimentary sequence target that were present in high copies number due to the rule of probability. As seen in Figure 12, when copies of the HIV ES added to the reaction were increased, amplification signal of internal control would decrease. According to the PCR result obtained from running a series of different concentrations of IC with 1000 copies of HIV ES in each reaction, there was a 1 Ct reading delay (equivalent to 2-fold difference) for the detection of HIV ES (FAM channel) when IC concentration increases to 500 copies/reaction (see Table 2). Therefore the amount of internal control to be spiked into each reaction was optimized to 100 copies per reaction where the Ct readings of the IC within the biological working range of the assay to be the same. With this being established, any delay in amplification of the internal control would signal the possibility of PCR inhibition, which would simultaneously delay the amplification of the HIV target.

Such situation would usually be observed as a later Ct reading in the amplification signal of the real time PCR.

Table 2. Ct readings of 1000 copies of HIV ES (FAM channel) with various concentrations of IC (Texas Red channel) spiked into the in-house HIV assay. IC was serially diluted from 100 copies to 1000 copies.

Internal Control Concentration (Copies/reaction)	Ct Readings	
	FAM Channel (Detection of HIV ES)	Texas Red Channel (Detection of IC)
100 copies	34.95	35.77
200 copies	34.90	34.43
300 copies	35.09	34.07
500 copies	35.94	32.91
800 copies	35.36	31.69
1000 copies	35.93	31.90

Figure 12. Real Time PCR Amplification plot of competitive PCR of HIV target and IC. Detection of 10-fold serial dilution of HIV ES (from 1 to 10^5 copies/ μ L) with 10 copies of IC using a common pair of primers. FAM- and Texas Red-labeled TaqMan probes (detected by Channel 1 & 3 respectively) were used to detect HIV ES & IC respectively.

Sources of PCR inhibitors might be found in the materials and reagents that come in contact with samples during processing or DNA purification. For instance, blood possessed lots of possible PCR inhibitor such as heme (Akane A, 1994), hemoglobin (Al-Soud & Radstrom, 2001), Immunoglobulin G (Al-Soud WA, 2000), anticoagulants like EDTA (Rossen L, 1992) and heparin (Izraeli, Pfliderer, & Lion, 1991). Excess potassium chloride (KCl), sodium chloride (NaCl) salts and ethanol (Löffert, Schaffrath,

Berkenkopf, & Kang, 1997) carried over from the extraction and purification process could also inhibit the PCR process. Therefore, to avoid false negative result, internal control is spiked into the PCR reaction to ensure that no PCR inhibition has occurred.

3.2.4 NIBSC HIV-1 standards

WHO international standard reagent [National institute of Biological Standards and Council (NIBSC)]: HIV-1 RNA 2nd International Standard (NIBSC code: 97/650), HIV-1 RNA Working reagent 2 (NIBSC code: 99/636) and HIV-1 RNA Working Reagent 3 (NIBSC code: 05/158), containing $5.56 \log_{10}$ IU/mL, $4.56 \log_{10}$ IU/mL, $2.56 \log_{10}$ IU/mL of HIV-1 respectively, were used as reference plasma to calibrate the HIV-1 ES. The HIV-1 ES was calculated to give a conversion factor of 1IU=0.54copies (Appendix A). A HIV-1 RNA genotypes, 1st International Reference panel (NCBIS code: 01/466) containing HIV-1 genotypes Group M (A, B, C, D, AE, F, G, AG), group N and Group O was used to determine the genotype inclusivity of the in-house assay (see Appendix B). These international standards were imported into Singapore and were extracted using the same extraction method as stated above with the patient samples in NUH-MDC lab with the BSL 3 facility.

3.3 Real time Polymerase Chain Reaction

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (qPCR) was used to amplify and simultaneously detect the quantity of the targeted DNA molecules using fluorophore dyes. PCR developed by Kary Mullis in 1983 worked on a basis of thermal cycling, which involves repeated cycles of heating and cooling of the reaction containing the DNA target, DNA polymerase, primers, deoxynucleosides (dNTPs) and buffer (K. Mullis, 1986). The thermal cycling includes denaturation, annealing and extension steps. Temperature was first increased to 95°C to denature and separate the double stranded DNA template to expose the DNA to its complementary primers. As temperature is lowered to the annealing temperature of the primers, the primers bind to the single-stranded target and DNA polymerase is attached and initiated the DNA synthesis. Next, the temperature is increased to the optimal extension temperature of the DNA polymerase depending on its type. During the extension step, DNA polymerase synthesized the DNA by incorporating dNTPs complementary to the template in the direction of 5'-phosphate group to the 3'-hydroxyl group of the dNTPs. The duration for each temperature step would be based on the recommendation of the kit's manufacturer, which would be provided based on the extension speed and performance of their proprietary's DNA polymerase. For an ideal amplification reaction, a single target would double with each amplification cycle. This thermal cycle would usually be repeated at 40 cycles before sufficient amount of DNA is produced for detection.

For real time PCR, a fluorophore dye is used to identify the amount of amplified product synthesized during each cycle. The fluorescence intensity of the fluorescent reporter dye signal was proportional to amount of amplified product present. At the end of each cycle, the fluorescence signal is captured by a detector in the qPCR thermocycler instrument and plotted against the cycle number on an amplification plot as seen in Figure 13. The amplification plot could be divided into three phases: exponential, linear, and plateau. During the lag phase (phase I), the fluorescence from the DNA amplification was undetectable above noise fluorescence. This phase was used to evaluate the baseline "noise" of the PCR amplification. Exponential amplification of the DNA was detected in phase II. Phase II was followed by a linear or plateau phase as reactants become exhausted (phase III). The point at which the fluorescent signal to be measured for the calculation of initial template quantity was during the amplification progress. Evident by Figure 14, the fluorescent signals collected at the exponential phase of amplification among the 96 replicated reactions were more reproducible, before limiting agents, accumulation of inhibitors, or inactivation of the polymerases have started to take an effect on the efficiency of the amplification (Gibson, Heid, & Williams, 1996; Heid, Stevens, Livak, & Williams, 1996). With this well-to-well reproducibility confirmed in the qPCR system, a calibrated standards dilution series with known concentration can be ran together with the unknown samples during qPCR for quantifying the unknown samples.

Figure 13. The 3 phases of the amplification plots are shown in linear scale: exponential, linear and plateau. The inserted equation describes the dynamics of the amplification during phase II. (Karlen, McNair, Perseguers, Mazza, & Mermoud, 2007)

Figure 14. 96 replicate reaction performed in a qPCR reaction showed the most reproducible region of amplification plot as the exponential phase. (Allen, N.D)

3.3.1 Real time PCR using TaqMan-based chemistry

TaqMan Probe-based real-time PCR required a pair of PCR primers just as regular PCR needed. On top of the PCR primers, this chemistry required a third oligonucleotide, with both a reporter fluorescent dye at the 5' end and a quencher dye at the 3' end attached. This is known as the dually-labeled fluorescent probe. TaqMan probes used the

Fluorescence Resonance Energy Transfer (FRET) quenching mechanism where quenching would occur as long as the reporter dye and quencher remains in close proximity of approximately 100Å (Richard A. Cardullo, 1988). Dark quenchers such as Black Hole Quenchers (BHQ) were used because they provided lower background fluorescence (Aldrich, N.D).

TaqMan probes were designed to anneal to one strand of the target sequence just slightly downstream of forward primer. In its free, intact form, no fluorescent emission can be measured as the fluorescent emission of the reporter dye is absorbed by the quencher dye. During the extension step of the PCR, Taq DNA polymerase would extend from the forward primer and would encounter the 5' end of the TaqMan probe. Taq DNA polymerase had an 5'-3' exo-nuclease activity, which would displace and degrade the 5' end of the TaqMan Probe as it extended, releasing the free reporter dye into the solution. Following the separation of the reporter dye and the quencher, signal was no longer quenched and therefore fluorescent signal could be detected from the reporter dye (see Figure 15). As each TaqMan probe was bound to a single DNA template during the PCR process, the fluorescent measured at each cycle is therefore directly correlated to the amount of DNA product synthesized (Heid et al., 1996).

Figure 15. TaqMan-based probe chemistry. TaqMan probe displacement with DNA polymerase extension released the reporter dye from the quencher, allowing quantitative fluorescent signals to be captured during the qPCR reaction.(Asuragen Inc, 2009)

3.4 Optimization of In-House Real time RT-PCR HIV-1 Viral load assay

3.4.1 Assay optimization

To ensure efficient and accurate quantification of the HIV target template, qPCR assays should be optimized and validated for quantitative range and specificity. This process required the use of an abundant and quantifiable control template. Hence the target was cloned into a recombinant plasmid, where a large pool of control template was achieved. This template would then be quantified by UV spectrometer. The criteria for the selection of the control template were as followed: it should be available in big abundance, it needed to be as similar as the unknown sample that was targeted in the assay and contained the sequence of interest, i.e. HIV gag gene for this in-house assay. Hence, for this research project where clinical HIV sample was the target interest, a HIV-positive, treatment naïve patient sample was selected and cloned as the external standard (ES) control using methods mentioned in previous chapter.

3.4.2 Annealing temperature Optimization

This in-house assay was optimized by first evaluating the annealing temperature of the newly designed primers, both forward and reverse using the ES as the control template. The annealing temperature of the primers would vary from the computed temperature obtained from the oligo design software, based the kind of quenchers used for the QPCR

chemistry. In this in-house assay, Black-Hole quencher (BHQ) was selected to quench the fluorophores. Black-Hole quencher would emit the energy they absorbed from the reporter fluorophore as heat rather than light of a different wavelength. This heat might influence the actual temperature during the assay run. From the oligo design software, which used the nearest neighbors' thermodynamic theory for the prediction of the primers' melting temperature, the melting temperatures of the forward and reverse primers gag183U/L were 61.25°C and 66.92°C respectively. Using those temperatures as a guide point, a range of annealing temperatures (60.3-72.3°C) was analyzed with the ES control template. A middle concentration of the external standard (1000 copies) was used to evaluate the annealing temperature. This would ensure that the fluctuations of the readings was not due to the detection limit of the assay and instead due to the varying annealing temperatures.

Duplicate points were also performed for each temperature to check for accuracy. Once the run was completed, the Ct readings and the last fluorescence (RFU) readings were examined. The temperature that gave the lowest Ct and highest RFU reading was deemed as the best performing condition. As observed in Figure 16a and 16b on the duplicate Ct readings of the HIV target obtained from the selected temperature range, 62.7°C gives the lowest Ct reading and the highest RFU reading respectively. However, taking into account that the clinical samples used in the assay contained human chromosomal materials, it was a precaution decision that the 2nd optimal and higher annealing temperature was selected to avoid non-specific binding of the primers. Therefore, 65°C was established as the assay's annealing temperature.

Figure 16. A) Ct reading trend of the primer annealing temperature gradient. Annealing temperature was varied from 60.3-72.3°C during the qRT-PCR run. B) Amplification plot of the HIV external control showing the florescence signal accumulated by each annealing temperature conditions as the amplification condition.

3.4.3 Primer concentration Optimization

After the working annealing temperature of the assay was established, the concentration of the primers, both forward and reverse had to be evaluated using the ES template control across a range of concentrations. Typically primer concentrations ranging from 0.1-0.9 μ M would be tested. To illustrate the primer concentration optimization strategy, Figure 17 shows the matrix map of the combination of forward and reverse primer concentrations from 0.1-0.4 μ M. For multiplex assay, a lower range of primer concentration was used. This was because a lower overall primer concentration could lower the chance of the priming reaction interfering with one another. A middle concentration of ES template control (1000 copies RNA) was used to assess each of the primer concentration combinational pairs. Using the previously established annealing temperature, these reactions were ran in duplicates in the presence of the TaqMan probes at 0.1 μ M.

		Reverse Primer [μ M]			
		<i>0.4</i>	<i>0.3</i>	<i>0.2</i>	<i>0.1</i>
Forward Primer [μ M]	<i>0.4</i>				
	<i>0.3</i>				
	<i>0.2</i>				
	<i>0.1</i>				

Figure 17. Diagrammatic representation of Primer concentration matrix. For each primer concentration combinational pair, qPCR reaction wells were performed with 1000 copies of ES control template with 0.1 μ M of TaqMan probe. Ct reading for each reaction well would be determined.

Once the runs were completed, the Ct readings and final fluorescence (RFU) readings of each primer concentration combinational pairs were examined. Based on the Ct results only (see figure 18), a combination of forward primer: 0.2 μ M with reverse primer: 0.3 μ M gave the lowest Ct reading but it had comparable results with other primer combination pairs. As qPCR reactions are performed in small volume of 25 μ L, it would be very sensitive to pipetting volume error. Therefore, a slightly higher primer concentration will overcome the insensitivity issue when insufficient reagents volume was being pipetted into the reaction. Therefore, the combination with 0.3 μ M of forward primer and 0.3 μ M of reverse primer was selected as the assay optimal primer concentration. This minute increment of primer concentration was still within the optimal

performance of the assay (± 0.5 Ct reading from the lowest Ct reading) and concurrently it was able to serve as a precaution for operator's errors.

Average Ct readings					
Reverse Primer [μM]					
Forward Primer [μM]	0.4	0.3	0.2	0.1	
	0.4	32.13	32.15	32.11	32.57
	0.3	32.48	32.12	32.27	32.99
	0.2	32.3	31.94	32.35	32.84
	0.1	32.78	32.53	32.47	32.94

Figure 18. The average Ct readings of 1000 copies of the ES control template amplified by the various combinations of forward and reverse primer concentrations that were performed in duplicates. The Ct reading in blue is the lowest generated Ct and those highlighted in yellow are readings ± 0.5 reading from the lowest Ct reading.

3.4.4 TaqMan Probe Concentration Optimization

After the optimal primer concentration was established, it was then necessary to determine the optimal TaqMan probe concentration for the in-house assay. Fluorescent probes such as TaqMan Probes used in qPCR assay typically would vary from the concentration of 0.1-0.5 μ M. Analysis of fluorescent probe optimization were similar to

that of the primer optimization. Using the previously established primer concentration and a middle concentration of ES template control (1000 copies), duplicate reaction wells were performed with the various concentration of the TaqMan probes. The probe concentration that generated the lowest Ct reading and highest final fluorescence signal (RFU) were then determined. However, for multiplex assay, a precaution should be practiced where the baseline fluorescent reading generated when using mixture of different fluorescent probe dyes. For each of the reporter dye, it should not be running too close to the background signal as this could cause problem with software baseline correction data analysis. Furthermore, the exponential phase of the amplification were required to be below the saturation fluorescent signal level of the instrument in order to get accurate result. Fluorescent signal level approaching the saturation level would cause problem such as spiking and poor signal uniformity. Figure 19 shows the Ct readings generated by the different concentration of TaqMan probes (0.1-0.5 μ M) were similar. However, it could be observed that with higher TaqMan probe concentrations, the baseline signal would approach the saturation level of 60,000RFU. Therefore the optimal probes concentration was established to be at 0.1 μ M.

Figure 19. The probe optimization data plotted as the raw fluorescence data against the PCR cycle number. Baseline signal was observed to increase with probe concentration. Average Ct readings of 1000 copies of ES control template with different probe concentration (ran in duplicates) were presented in the embedded table.

3.4.5 Polymerase Chain Reaction Assay Set-up protocol

The in-house HIV viral load assay was performed using Superscript™ III Platinum® One-Step Quantitative RT-PCR System Master Mix reagents (catalog no. 11732; Invitrogen, USA) in total 25 μl reaction volume containing 10 μl of RNA sample, 100 copies of IC molecule, forward/ reverse primers at a final concentration of 0.3 μM and

both probes at 0.1 μ M each. The concentrations of primer and probes had been optimized using the ES control template prior the validation runs with the patient samples were mentioned in previous chapters. A master mix containing all reagents, except the RNA samples would be prepared and 15 μ L of the master mix pipetted into each reaction well before pipetting the individual samples into each wells.

Thermal cycling was performed by Stratagene Mx3000P thermocycler (Stratagene, La Jolla, USA) using the following steps: reverse transcription at 55°C for 30 min and initial denaturation at 95°C for 2.5 min, followed by denaturation at 95°C for 17 s and annealing at 65°C for 31 s and extension at 68°C for 32 s. The amplification cycle was repeated 48 times. The increase in the fluorescent signal was registered during the annealing step of the reaction. Fluorescence detection was read at the 65°C annealing step of each cycle (see Figure 20). The temperatures of the annealing step had been optimized to ensure maximum amplification efficiency. The duration and temperature of the other steps during the reaction followed the recommendation by the manufacturer's protocol of the Invitrogen Superscript™ III kit, which is based on the activation duration, conditions and extension speed of the proprietary Platinum® Taq DNA Polymerase for optimal specificity, consistency, and efficiency (Life Technologies Inc., 2011).

Figure 20. Stratagene Mx3000P thermocycler (96-wells format) and the RT-PCR thermal profile for the in-house viral load assay protocol.

The adaptive baseline mode of the Mx3000 qPCR software was used for cycle threshold (Ct) determination for data analysis for both the target and IC. Whenever necessary, manual adjustment of the cycle threshold would be performed to account for background fluorescence. Single determination to mimic routine clinical use, were performed for the individual patient samples. The Mx3000P thermocycler is a 96-well format, where 96 samples could be run concurrently in a single assay, of which 16 reaction wells were used for external standards and non-template control (NTC); the remaining wells had allowed a maximum of 80 patient samples to be analyzed in a single run. Maximizing the number patient samples per run helped to further lower the cost of the assay per sample. Each assay run included 2 negative controls to exclude contamination. The first negative control would be assigned to the first reaction well and the other would be assigned to the last reaction well when the master mix were pipetted into the 96-wells. The purpose of

the first negative control was to check that the master mix prepared was contaminant-free; the latter was to check for cross contamination during sample loading.

Duplicate standard curves generated from the ES were used to determine the copies of HIV-1 RNA per reaction. Figure 21 shows the amplification plot of the serially diluted ES. An ES regression standard curve would be plotted using the known ES concentration against their respective Ct readings, an equation of the best fit-line was then generated (see Figure 22). The amount of starting material in the patient samples were determined by the interpolation of the unknown samples' Ct readings against the ES calibration curve. Final concentration of viral RNA copies per ml was calculated for each sample with the sample volume used from the eluted volume taken into account. Since during sample preparation step, HIV viral in 1mL of blood plasma was concentrated and extracted into a volume of 50 μ L, and of which, 10 μ L of the extracted RNA was used in the qPCR assay. This 10 μ L RNA used would contain RNA equivalent to that in 200 μ L of plasma. Therefore, the amount of RNA copies obtained from the assay had to be multiplied by 5 to obtain the actual viral copies in 1 ml of blood plasma (copies/mL). The determined Ct value of the IC was used to check for PCR inhibition. A sample with a Ct value of the IC delayed by more than 2 cycles compared to that of equivalent ES was considered invalid due to PCR inhibition and repeated.

Figure 21. Amplification plot of 10-fold serial diluted external standards (ES) with concentrations from 1 to 10⁶ copies/μL that were ran simultaneously with each batch of clinical sample. Ct readings of these ES were used for the generation of calibration standard curve.

Figure 22. A standard curve generated from the Ct readings of the known concentration of external standards (ES) that were serially diluted. Ct readings of unknown samples were then interpolated on the curve using the best-fit equation.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Non-Clinical Performance Evaluation

To determine the performance of the in-house HIV viral load quantification assay, the following performing factors were considered and determined: linearity of the dynamic range, analytical sensitivity, precision and specificity of the in-house assay. Due to the limitation amount of the NIBSC international standards, the external standards (ES) were used to determine these non-clinical performance factors. The ES had been calibrated against the NIBSC international standard to give a conversion factor of 1IU of NIBSC international standards is equivalent to 0.54 copies of ES.

4.1.1 Linearity of dynamic range

The linear dynamic range of a detection assay is defined as the maximum linear response divided by the background noise. Linearity of the dynamic range of the in-house assay was established based on the mean value of the Ct values that were determined from the replicate assays. Each of the six assays was applied to eight serially diluted ES control template from three different dilution lots. These values were plotted against their input copy number of the ES in a log-scale. Coefficient of the linear regression of the dynamic ranged from 10 copies per reaction to 10^8 copies per reaction (corresponding to 50 copies/mL to 5×10^8 copies/mL of plasma sample) was $r^2=0.999$ (see Figure 23). This

indicated that the assay is linear over its quantification range. For this dynamic range of quantification, the RT-PCR efficiency was as high as 95.6%.

Figure 23. Linear relationship of in-house viral load assay of serially diluted external standards (ES) from 3 different dilution lot was established from 10 copies per reaction to 10^8 copies per reaction with coefficient of regression of 0.999.

4.1.2 Analytical sensitivity

“Analytical sensitivity” represents the smallest amount of substance in a sample that can accurately be measured by an assay (Saah & Hoover, 1997). Analytical sensitivity of the in-house assay was established using serial dilution of ES control template from 3.2×10^3 copies per reaction down to 1 copy per reaction (corresponding to 1.6×10^4 copies/mL to 5

copies/mL of plasma). The serially diluted ES were tested in 4 replicates within a run, with 4 runs being performed. Probit Analysis is a specialized regression model of binomial response variables. Probit regression was used to determine the projected response rate according to a dose response model. It transformed the sigmoid dose-response curve to a straight line that could then be analyzed by regression either through least squares or maximum likelihood. As seen in Figure 24, at a concentration of 12.3 copies per reaction (corresponding to 61.5 copies/mL plasma), detection probability was 95% or higher (49.5; 89.5 copies/mL).

Figure 24. Probit regression to determine the limit of detection (LOD) of the In-House assay at 61.5 copies/mL with 95% confidence intervals (dotted lines show the 95% confidence interval).

4.1.3 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (Taylor, 1999). Ideally a measurement device should be both accurate and precise, with all the measurements close to and tightly clustered around the known value. Determination of the in-house assay's precision was performed at two levels: intra- and inter-assay precisions.

Intra-assay precision expresses the precision under the same operating conditions over a short interval of time. Intra-assay precision is also termed as repeatability. The intra-assay precision was determined by a single operator, performing the assay with the same tube of ES template control stock from the same dilution lot. Intra assay precision of the ES containing known $7.0 \log_{10}$ copies/ μL (corresponding to 5×10^8 copies/mL plasma) with 8 replicates were tested in the single experiment seating. The mean of the calculated copy number was determined as $6.92 \log_{10}$ copies/ μL , with coefficient of variation (CV) of 0.825% and standard deviation (SD) of $0.04 \log_{10}$. To ensure that the in-house assay is able to accurately quantify viral load at its detection limit, the ES control template was diluted to $1.0 \log_{10}$ copies/ μL (corresponding to 5×10^2 copies/mL plasma) and assayed in 8 replicates in single experiment seating. The mean of the measured valued was $0.5 \log_{10}$ copies/ μL , with CV of 2.2% and SD of $0.23 \log_{10}$. Therefore, regardless of the extremely high or low amount of target, the in-house assay would be able to perform at high precision within a single run.

Inter-assay precision expresses intra-laboratory variations: different days, different preparation lots of the serially diluted ES control template. Inter-assay reproducibility was obtained by performing six independent experiments, CV% of Ct reading was obtained as less than 4.2% and SD less than 0.9 cycles for all the ES that were serially diluted from 10 copies/reaction to 10^8 copies/reaction (refer to Table 3). This shows that the inter assay precision of the in-house assay had less than 2-folds difference for its lot-to-lot variations.

Table 3. Inter-assay variability of the in-house assay using external standards (ES).

HIV ES dilution (copies/reaction)	Ct Mean readings	SD	CV%
10^8	14.54	0.47	3.21%
10^7	18.16	0.76	4.19%
10^6	21.64	0.70	3.25%
10^5	25.26	0.46	1.83%
10^4	28.84	0.90	3.14%
10^3	32.70	0.59	1.80%
10^2	36.27	0.63	1.74%
10^1	39.99	0.90	2.26%

4.1.4 HIV-1 Genotype Inclusivity

HIV-1 genetic diversity is a key challenge to the development of HIV-1 quantification assays (Rouet et al., 2007a). Therefore, subtype inclusivity was evaluated using the HIV-1 RNA genotypes, 1st International Reference panel (NCBIS code: 01/466) that consisted of HIV-1 genotypes Group M (A, B, C, D, AE, F, G, AA-GH), group N and Group O. Among the genotypes in the NIBSC genotype panel, the in-house assay was successful in detecting all of the genotypes of group M (A, B, C, D, AE, F, G, AG), and group N. Group O was not detected from the panel (see Table 4). However, this was insignificant in the clinical application of Singapore HIV-1 patient cohort as the dominant genotypes are A, B and AE. Each of them formed about 45% in a study of recent sero-converters (Hsu, Subramaniam, Bacheler, & Paton, 2005; Kalish et al., 2002; C. C. Lee, Sun, Barkham, & Leo, 2009). As the viral copy numbers of the NIBSC genotype standards were not given; therefore detection sensitivity of each individual genotypes could not be determined. The incapability in detecting group O of the WHO Reference Panel was a common feature of many in-house developed HIV-1 assays (Rouet et al., 2007a).

Table 4. Detection of HIV-1 genotypes of Group M (A, B, C, D, AE, F, G, AA-GH), and Group N with their Ct readings by the in-house HIV viral load assay.

WHO HIV-1 RNA genotypes	Result
(1st International Reference panel)	Ct readings (dR)
A	35.72
B	38.58
C	35.51
D	34.54
E	34.78
F	34.8
G	34.37
AG	33.63
N	36.3
O	No Ct (Not Detected)

4.2 Clinical evaluation of in-house assay with 329 patient samples

Plasma samples from 329 HIV-1 patients on active follow-up were evaluated with the on-house assay in comparison with the CTM and ART assays. Using a cut off quantifiable level of 50 copies/mL for the commercial and in-house assay, 227 samples were quantified with viral load >50 copies/mL by both the in-house and commercial assays (CTM: n=119; ART: n=108)). Agreement between the assays was assessed using a Bland-Altman model (Bland & Altman, 1999) as seen in Figure 25. The mean difference of the computed viral load between $\log_{10}$ -transformed values of the in-house and CTM assays was -0.094 (95% of values between -1.18 to 0.99). Corresponding values for the in-house and ART assays were 0.056 (95% of values between -0.83 to 0.94). According to the Bland-Altman analysis, among the $\log_{10}$ -paired differences, 69% of the samples tested in both comparisons were within $0.5\log_{10}$. 25% of the in-house/CTM viral load comparison cohort and 30% of the in-house/ART viral load comparison cohort were within the difference interval of 0.5 to $1.0\log_{10}$; 6% of the in-house/ CTM and 1% of the in-house/ ART showed greater than $1.0\log_{10}$ difference.

The Bland-Altman analysis is a measure of the agreement between two instruments measuring on a continuous scale. In the current analysis, no funneling effect was observed in both of the Bland-Altman plots. This showed that the differences in viral load reading between the in-house and commercial assays were evenly distributed across the range of average values, thereby supporting no bias in differences between high versus low quantification values. Moreover, The linear regression R-values of the In-house/

CTM and the in-house/ ART comparison were 0.79 and 0.90 respectively. Regression analysis revealed no statistically significant correlation between differences in $\log_{10}$ -transformed values with average $\log_{10}$ values in the Bland-Altman plot. A similar assessment in South Africa using 458 specimens revealed 87% of samples falling within $1\log_{10}$ difference (Rekhviashvili et al., 2007).

Figure 25. Bland-Altman plots of valid quantification results for clinical plasma samples.

Graph A shows comparison of In-House and CTM (n=118). Graph B shows comparison of in-house and ART (n=97).

The in-house assay has demonstrated comparable clinical performance with recently large-scale evaluated in-house assays currently in clinical use. Dorsten et al. validated an in-house assay against 4 commercial assays on 1500 patient samples with a mean difference of paired viral loads of $0.31 \log_{10}$ (Drosten et al., 2006b). Rouet et al conducted comparative evaluations of an in-house protocol in 898 samples against 2 commercial assays and determined mean differences between 0.1 to $0.35 \log_{10}$ (Rouet et al., 2007b). The current mean difference of $-0.094 \log_{10}$ and $0.056 \log_{10}$ were compared favorably. Previous comparisons between the ART and CTM assays had shown a mean $\log_{10}$ differences in the viral load readings from -0.24 to 0.51 (Braun et al., 2007; Foulongne, Montes, Didelot-Rousseau, & Segondy, 2006; Gueudin et al., 2007; Schutten et al., 2007). This could explain the difference in results noted between comparisons with the ART and CTM in this research study.

Frequency distribution of the in-house, CTM and ART viral load readings reflected similar unimodal distribution in pair-wise comparison (see Figure 26). For the 119 samples comparing In-House and CTM assay, the modal peaks were 3.68 and $3.79 \log_{10}$ respectively; for the 108 samples comparing In-House and ART assay, the modal peaks were 3.49 and $3.40 \log_{10}$ respectively. To date, no gold standard test has been established for the viral load quantification of HIV-1 (Daar et al., 2001; Owens et al., 1996). Hence, the difference between the in-house assay and CTM was not further investigated.

Figure 26. Histogram of the log₁₀ viral load values for the in-house, COBAS TaqMan HIV-1 and Abbott RealTime HIV-1 assay. Graph A shows the comparison between in-house and CTM (n=119). Graph B shows the comparison between in-house and ART (n=108).

4.2.1 Diagnostic Sensitivity and Specificity at clinically relevant 200 copies/mL cutoff

In a clinical setting, the use of diagnostic sensitivity and diagnostic specificity is one of the approaches to quantify the diagnostic ability of the test. Diagnostic sensitivity is the proportion of true positives that were correctly identified by the assay, whereas diagnostic specificity is the proportion of true negatives that were correctly identified by the assay. A 2 by 2 table was used to reflect the results after both the in-house assay test and the commercial established assay test have been performed on a pre-determined population consisting of people with the detectable and non-detectable HIV viral load.

To evaluate clinical sensitivity and specificity, the performance of the in-house assay was evaluated against the CTM and the ART assays at a cut-off of 200 copies/mL. Detectable viral load above 200copies/mL was considered as positive in the diagnostic sensitivity and specificity calculation. Studies have showed that a maintenance of viral load below 200 copies/mL was sufficient for viral suppression in clinical setting and viral load below detectable level might not have much of clinical significance (Combescure C, 2009; Phillips et al., 2001; Pierre-Marie Rogera, 2001). Against the CTM assay, the sensitivity was 99.1% and specificity 100%. Only 1 sample of the 118 samples reported as more than 200 copies/mL by the CTM assay was reported below 200 copies/mL by the in-house assay. Against the ART assay, the in-house assay had a sensitivity of 96.8% and a specificity of 96.4%. 92 samples were reported by both assays with viral load more than 200 copies/mL. 4 samples were reported positive at this cutoff by the in-house but

negative by the ART and 3 samples negative by in-house but positive by ART (see Table 5). Assay inhibition was detected in only 1 clinical sample and the repeat result was used in this analysis.

Table 5. The 2x2 table representations of the diagnostics sensitivity and specificity of in-house viral load assay against COBAS TaqMan HIV-1 and ABBOTT RealTime HIV-1 assay.

		In-house HIV Viral Load assay	
		Positive (>200 copies/mL)	Negative (<200 copies/mL)
	Results		
COBAS TaqMan HIV-1 assay	Positive (>200 copies/mL)	118	1
	Negative (<200 copies/mL)	0	32

		In-house HIV Viral Load assay	
		Positive (>200 copies/mL)	Negative (<200 copies/mL)
	Results		
ABBOTT RealTime HIV-1 assay	Positive (>200 copies/mL)	92	3
	Negative (<200 copies/mL)	4	79

The most extensively validated in-house assays developed by Lima et al. have lower limit of detection ranging from 50 copies/mL to 400 copies/mL, similar to this in-house assay. Using quantification cut-off of viral load around 2.0 log₁₀ (100 copies/mL) could result in poorly correlation as shown in a recent comparison study between the new CTM and the older Roche COBAS HIV-1 Ampliprep Amplicor, where the kappa statistic between the

two assays was only 0.19 at the detection limit of 40 copies/mL (Lima, Harrigan, & Montaner, 2009). Evaluation of performance characteristics at these low values might result in artefactual differences. In the current analysis, samples showing discordance at a cut-off of 200c/mL were all detected by the in-house use, albeit at lower values. These values would be reported as being less than 200 copies/mL, but detectable. Clinically, transient elevations of viral load above 50c/mL have been defined as “blips” which were unassociated with treatment failure or resistance emergence (P. K. Lee, Kieffer, Siliciano, & Nettles, 2006).

CHAPTER 5. CONCLUSION

HIV-1 is a global pandemic with the most recent World Health Organizations' report in November 2009 estimating 33.4 million infections worldwide and 2.0 million deaths in 2008 (UNAIDS, 2009a). An estimated 7 million individuals in developing countries would be on therapy by 2010 (Katabira & Oelrichs, 2007). Diagnosis is a central theme in HIV control, not only for the management of the patients, but also the detection of contact cases. HIV-1 viral load is essential for treatment monitoring. No surrogate marker has been able to replace the viral load for monitoring treatment response (Calmy et al., 2007). However, the majority of HIV-1 infected persons reside in developing countries where access to viral load monitoring is limited due to the need for extensive laboratory facilities, trained personnel and financial cost (UNAIDS, 2009b). In Singapore, a developed country, great support were given by the government to ensure that drug treatment for the HIV patients are greatly subsidized and affordable for the patients (Ministry of Health Singapore, 2002). However, till today, the cost for viral load monitoring is borne by patients. The payable cost per assay is approximately US\$150. Due to this high cost, the average frequency of viral load monitoring is 0.7 viral loads per patient per year, a gross underutilization of an essential treatment-monitoring tool. To overcome this limitation, an in-house HIV-1 viral load real time RT-PCR assay was developed and validated in this research project, aimed to provide an inexpensive monitoring tool for the HIV patients.

In this study, the developmental evaluation of this in-house HIV-1 viral load assay was described using 327 plasma specimens. This duplex assay utilized the Stratagene Mx3000P real-time qPCR platform and FAM- and Texas Red- labeled TaqMan probes for specific detection of the HIV-1 gag-gene region and internal control molecules respectively. The external standard (ES) control template for quantification calibrated against the NIBSC international standard, was cloned from a HIV-positive patient sample. The design of this ES control template for this in-house assay overcame the need of a high maintenance cost Biosafety Level 3 laboratory for virus culture of quantification standards. Two previous studies from Thailand, where subtypes AE and B predominated, have described clinically validated in-house assays with 105 and 50 HIV-1 clinical samples respectively (Promso et al., 2006; Watanaveeradej et al., 2005). However, both assays lacked internal control, an important feature in molecular test as inhibition rates could be as high as 3.7% for large-scale validations (Drosten et al., 2006a). In this research study, inhibition was noticed in 1 patient sample with the aid of the internal control that was spiked into each reaction well. This sample was then re-extracted and re-analyzed.

Despite the great genetic diversity of HIV-1, equal detection and quantification of Human Immunodeficiency Virus-1 (HIV-1) (Group M and N) in human plasma specimens had been demonstrated by this in-house assay using the WHO Reference Panel. At a concentration of 61.5 copies/mL, detection probability was 95% or higher (95% confidence interval, 49.5-89.5 copies/mL). Other than a wide dynamic range of 5x8.0 log₁₀ copies/mL, it was shown to have a good intra-assay precision with coefficient of

variation (CV) of 0.825% and standard deviation (SD) of 0.04 $\log_{10}$ with 8.0 $\log_{10}$ copies per reaction of ES (corresponding to 5×10^8 copies/mL plasma) and with CV of 2.2% and SD of 0.23 $\log_{10}$ for 2.0 $\log_{10}$ copies per reaction of ES (corresponding to 5×10^2 copies/mL plasma). It has also been demonstrated to produce inter-assay precision, with CV% of Ct value less than 4.2% and SD less than 0.9 cycles for all the external standards of serial dilution from 10 copies/reaction to 10^8 copies/reaction (corresponding to 50 copies/mL to 5×10^8 copies/mL plasma). Inter- and intra- assay variability of less than 0.5 $\log_{10}$ copies has been abided by clinicians to be of no clinical significance.

This in-house assay is further evaluated against 2 current commonly used commercial assays by quantifying the viral load of more than 300 HIV-positive patient samples. The Bland-Altman analysis showed good agreement between the in-house and both commercial assays for 96% of the samples. The mean difference between $\log_{10}$ -transformed values of the in-house and CTM assay was -0.094 ([-1.18; 0.99], CI 95%). Corresponding values for the in-house and ART assay were 0.056 ([-0.83; 0.94], CI 95%). The marginal differences in viral reading generated by in-house and commercial assays gave sufficient evidence that this in-house assay is capable to perform as well as existing commercial kits and be a alternative solution for being a cheaper replacement viral load test for the patients.

Today, tests of diagnostic and disease monitor contribute to overall cost saving in healthcare system. In the new era of medical diagnostic, it no longer involves just detection and therapy. It has evolved into a whole process of assessment of disease risks,

early detection of illness, with support to individual therapy selection based on individual condition. Beyond these, a monitoring system is vital to determine the efficiency of the therapy that the patients received. For the HIV patients, viral load testing is an essential monitoring tool for the disease progression. In conclusion, the in-house HIV-1 viral load assay that was developed is a reliable, easy to use, affordable assay to increase access to reliable monitoring of response to therapy in regions where subtype B and AE predominate. The total cost of all the reagents was less than US\$10 per reaction. This assay is currently being implemented into routine testing in Singapore.

CHAPTER 6. FUTURE WORK

Currently, this in-house assay is being used in Tan Tock Seng Hospital's Infectious Disease Research Laboratory in the clinical trial phase. The detection kits are assembled in the author's laboratory. For any routine assay used in clinical setting, consistency and reproducibility of each batch of kits being produced is crucial. There is a need for continuous monitoring of the batch-to-batch consistency of the primers and probes' performance and the quality and quantity of the ES produced. The possibility of signing up with a well-recognized quality control program should be considered in the future. Together with this, the quality and performance of the in-house assay kit in the clinical setting can be monitored. With this assay developed as an in-house kit, it is essential to ensure that the quality of kit being produced in the laboratory is consistent and maintained at high quality. Methods of this in-house kit's quality assurance can be further studied.

With the availability of the inexpensive in-house viral load assay, continued surveillances of the HIV cohort can be carried out. This will promote up-to-date reporting of the situation of the patients, and eventually gaining further understanding of the HIV cohort for other research areas such as HIV assay and HIV drug development and drug resistance. With a well-established treatment monitoring system, the information on the transformation of HIV virus obtained will be helpful in generating hypothesis for future research to help the HIV scientific community. This continuous surveillance allows the monitoring of the mutation pattern of this highly mutating HIV virus, which enables

researchers to constantly develop updated detection assay based on the prognosis of the HIV metamorphosis.

Numerous efforts to increase accessibility of antiviral drugs for HIV patients have been made by international organization in the recent years. However, effort to increase accessibility of the viral load monitoring is limited. A point-of-care (POC) device can be explored to reach out to the HIV-1 patients with limited access to the central laboratories, where they are able to monitor their viral load at the convenience of their own homes. The in-house assay has been applied on a prototype portable platform. A prototype device using this in-house assay was presented at the 2009 International AIDS Society meeting in South Africa (Ng et al., 2009). In brief, the portable, credit-card-sized real-time device using this in-house viral load assay protocol described here was demonstrated to accurately quantify HIV-1 cDNA with a r^2 value of 1.00 compared to the bench top size Stratagene Mx3000P real-time PCR system (Stratagene, La Jolla, USA). This report has also shown the repeatability and reproducibility of this in-house HIV-1 viral load assay. With this evidence, a viral load mathematical model can be developed, where the samples can be tested without the need of running a series of external quantification standards to serve as a calibrating standard curve to determine the viral load. This mathematical model can be evaluated by comparing the viral load results derived from the mathematical model and those obtained by the protocol discussed in this report. A comparative analysis will be performed and discussed in the near future. By eliminating the need to run calibrating ES with the patient samples, it will further enhance the possibility of a point-of-care HIV-1 viral load device to the patients at a lower cost. This will therefore serve as

a solution to increase patients' accessibility to treatment monitoring tool at an affordable cost.

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APPENDIX

Appendix A. Calibration of external standards with NIBSC standards.

Objective: To compare the Ct values of the external standards (ES) against the NIBSC 97/650.

Note:

- 1) ALL reactions at **25ul** volume
- 2) Sample aliquot: **10ul**
- 3) NTC: tRNA (0.01ng/ μ L)
- 4) **65°C** Annealing temperature
- 5) **100** copies of IC added per well

Results:

Treating serially diluted NIBSC standard samples as unknown and used the ES standard curve to determine the copy number of the NIBSC standards. A correlation curve is plotted and the adjustment factor was determined as **2.49** based on the best-fit equation.

Correlation between NIBSC standards against External standards(ES)

Appendix B. NIBSC Imported standards.

Blanche Lane
South Mimms
Potters Bar
Hertfordshire EN6 3QG
United Kingdom

Tel: +44 (0)1707 641000
Fax: +44 (0)1707 641050
Email: enquiries@nibsc.ac.uk
Web: <http://www.nibsc.ac.uk>

Working Standard HIV-1 RNA Working Reagent 3 NIBSC code: 05/158-xxx Instructions for use (Version 2.0, Dated 04/03/2008)

1. INTENDED USE

This material is not for in vitro diagnostic use.

This is a newly formulated working reagent, manufactured with a low value intended for use in ultra sensitive assay systems. It has been produced using the same virus and diluent as has been used for the production of our Working Reagents 1 and 2. It is a replacement for the low copy number Working Reagent 99/766 that has now been withdrawn. It is intended for use in conjunction with diagnostic test systems / assays that detect HIV-1 RNA to monitor the performance of these systems. It can be used to monitor the consistency of test performance using statistical process control on a daily basis and, over a period of time, as a retrospective monitor of batch performance. The use of these products will indicate whether the sensitivity of an assay system in routine use is varying with time or with batch. It is for the user to determine limits from the use of our products and whether our products should be detected in every series of tests and the appropriate steps to take if our products are not detected or less well detected than previously. The products are not designed to provide any direct clinical data on any given specimen. These reagents should be used in addition to the batch specific positive and negative controls supplied with each batch of kit by its manufacturer. Our products are supplied for use in their final form and users are instructed not to further dilute the material other than as required in individual test procedures.

2. CAUTION

This preparation is not for administration to humans.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

This Working Reagent 3 has been produced with 10 fold less virus than the HIV-1 RNA Working Reagent 1 (WS-1) (99/634-***), it is therefore expected to have a value of 2.56 IU (log₁₀)/ml. The Working Reagent 3 has been assayed in triplicate in three independent assays in conjunction with the International Standard for HIV-1 RNA. The working reagent is considered acceptable if the overall geometric mean falls within the range: 1.99 – 2.80 Log₁₀ IU/ml.

4. CONTENTS

Country of origin of biological material: United Kingdom.
The HIV-1 RNA Working Reagent 3, code 05/158 contains ~1.1ml of liquid and consists of an HIV-1 genotype B virus diluted in pooled human plasma. The human plasma is supplied by the UK National Blood Service (NBS) and all packs have been pre-screened by the NBS, prior to dispatch to NIBSC, and tested negative for the following markers: HBsAg, anti-HCV, anti-HIV, HCV RNA and anti-HTLV.

5. STORAGE

This Working Reagent should be stored at colder than -65°C.

6. DIRECTIONS FOR OPENING

Vials have a screw cap; an internal stopper may also be present. The cap should be removed by turning anti-clockwise. Care should be taken to prevent loss of the contents. Please note: If a stopper is present on removal of the cap, the stopper should remain in the vial or be removed with the cap.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the material

The user should follow the assay kit manufacturer's instructions. The HIV-1 RNA Working Reagent 3 should be used in addition to the positive and negative controls supplied with each lot of kit and treated as if it were a routine test sample. Only the controls supplied with each lot of kit by the manufacturer are used to determine the validity of assays and to calculate the cut-off value that forms the basis for donor screening or diagnosis. The undiluted reagent should react positively in most NAT assays.

THIS MATERIAL IS SUPPLIED FOR USE IN ITS FINAL FORM AND SHOULD NOT BE FURTHER DILUTED OTHER THAN AS REQUIRED IN INDIVIDUAL TEST PROCEDURES.

Users should be aware that different batches of the same assay kit and/or different assay conditions may give different values with this reagent.

8. STABILITY

NIBSC follows the policy of WHO with respect to its reference materials.

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Upon receipt, vials of the HIV-1 RNA Working Reagent 3 should be stored at colder than -65°C. The stability of this material is being monitored by NIBSC. The stability of previous batches of the Working Reagents have not lost potency when stored below -65°C for periods of up to 7 years. The shelf life assigned to this working standard will be 5 years when stored at colder than -65°C.

Users who have data supporting any deterioration in the characteristics of any Working Standard/Reagent are encouraged to contact NIBSC.

A freeze-thaw indicator is included in the package along with interpretation instructions. If the freeze-thaw indicator shows that the reagent has been completely or partially thawed, it should not be used. If this occurs, please contact NIBSC and provide time/date of receipt of package and condition.

9. REFERENCES

None

10. ACKNOWLEDGEMENTS

N/A

11. FURTHER INFORMATION

Further information can be obtained as follows;

This material: enquiries@nibsc.ac.uk

WHO Biological Standards: <http://www.who.int/biologicals/en/>

Derivation of International Units:

<http://www.nibsc.ac.uk/products/faq.asp>

Ordering standards from NIBSC:

<http://www.nibsc.ac.uk/products/faq.asp>

NIBSC Terms & Conditions: <http://www.nibsc.ac.uk/terms.html>

National Institute for Biological Standards and Control

**WHO International Standard
HIV-1 RNA Genotypes, 1st International Reference Panel
NIBSC code: 01/466
Instructions for use
(Version 4.0, Dated 04/03/2008)**

1. INTENDED USE

HIV-1 exhibits substantial genetic diversity and several different genotypes of HIV-1 exist. There is a major group (group M), consisting of subtypes A-K, and a more diverse collection of outliers have been referred to as groups N and O. Many of the early nucleic acid-based tests (NAT) had a fairly narrow band of specificity targeted mainly at subtype B viruses, as these predominated in the Western World. Greater awareness of the HIV genetic diversity and the desire to detect as many strains of HIV as possible has led to a number of improvements in assay design. However, it has been recognised that some assays are still poor at detecting certain subtypes, occasionally giving low or negative results for samples that are clearly positive in other assays.

The WHO agreed that there is a need for a well characterised reference panel of different HIV-1 subtypes for use in regions of the world where non-B subtypes of HIV-1 predominate and by laboratories involved in NAT diagnosis and by kit manufacturers. The 1st International Reference panel for HIV-1 RNA (Code 01/466) is provided for use in qualitative and quantitative HIV-1 RNA assays.

The Panel consists of 11 samples representing different HIV-1 Genotypes (A, B, C, D, AE, F, G, AA-GH, group N and group O) as well as a negative diluent control.

This panel has been evaluated in an international collaborative study and a report of this study has been submitted and accepted by the WHO Expert Committee on Biological Standardisation (ECBS). At the recommendation of the ECBS, this panel has been established as the 1st International Reference Panel for HIV-1 RNA Genotypes.

2. CAUTION

This preparation is not for administration to humans.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

There is **no unitage assigned to this panel.**

4. CONTENTS

Country of origin of biological material: United Kingdom.

The panel contains infectious HIV-1 and must only be handled in appropriate containment facilities by fully trained and competent staff and in accordance with the local national safety guidelines (such as the UK "Protection against blood-borne infections in the workplace: HIV and hepatitis", Advisory Committee on Dangerous Pathogens, HMSO, London).

5. STORAGE

The Biohazard container contains a panel of 10 viruses of different subtypes/groups and one negative control, following receipt all vials should be held in a freezer below -65°C.

6. DIRECTIONS FOR OPENING

Vials have a screw cap; an internal stopper may also be present. The cap should be removed by turning anti-clockwise. Care should be taken to prevent loss of the contents. Please note: If a stopper is present on removal of the cap, the stopper should remain in the vial or be removed with the cap.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the material

The vials should be rapidly thawed just before use, and well mixed by inverting or briefly vortexing (5 seconds). The subtype panel consists of 11 vials (code 01/466). The panel members should be tested in accordance with the assay protocol. We anticipate that vials will be used only once.

We do not recommend use after a freeze thaw cycle.

The materials are provided solely for the purposes described within the document. They will be discarded after use and will not be used for any other purpose.

8. STABILITY

NIBSC follows the policy of WHO with respect to its reference materials. It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

ECBS report ref: WHO/BS/03.1961
http://whqlibdoc.who.int/hq/2003/WHO_BS_03.1961.pdf

10. ACKNOWLEDGEMENTS

N/A

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.ac.uk
WHO Biological Standards: <http://www.who.int/biologicals/en/>
Derivation of International Units:
<http://www.nibsc.ac.uk/products/faq.asp>
Ordering standards from NIBSC:
<http://www.nibsc.ac.uk/products/faq.asp>
NIBSC Terms & Conditions: <http://www.nibsc.ac.uk/terms.html>

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.ac.uk

**WHO International Standard
HIV-1 RNA, 2nd International Standard
NIBSC code: 97/650
Instructions for use
(Version 3.0, Dated 04/03/2008)**

1. INTENDED USE

The 1st International Standard for HIV-1 RNA, NIBSC code 97/656, was established by the WHO Expert Committee on Biological Standardisation (ECBS) in 1999 and has since been extensively used for assay validation and to calibrate secondary standards and working reagents. Diminished stocks and the discovery that it contains HBV DNA led to the further evaluation of a candidate standard used in the original study as a possible replacement. This was based on a genotype B (env V3, gag) field isolate of HIV-1 provided by Dr P Simmonds, University of Edinburgh, UK. The virus was isolated post-mortem from a patient that had died from an AIDS-defining illness, was propagated on PBMCs and a low passage stock stored down. The virus was diluted in pooled human cryosupernatant and freeze-dried. Eight laboratories from 6 different countries participated in a collaborative study to evaluate the replacement for the 1st International Standard for HIV-1 RNA. A total of eight different NAT were used, five in a quantitative format and three qualitative, and five were commercial assays. A report on the study was considered by the WHO ECBS in November 2005 and the material, NIBSC code 97/650, was established as the 2nd International Standard for HIV-1 RNA.

2. CAUTION

This preparation is not for administration to humans.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

A unitage of 5.56 log₁₀ IU /vial has been assigned to this material.

4. CONTENTS

Country of origin of biological material: United Kingdom.

Each vial contains 1ml of lyophilized human plasma cryosupernatant containing HIV -1 virus in a 2ml crimp top vial.

5. STORAGE

The vial should be stored at -20°C on receipt; each vial is intended to be used only once.

6. DIRECTIONS FOR OPENING

Vials have a 'flip-up' circular cap. Either on the cap or the collar of the vial, there is an indication of the point at which to lever off the cap. This exposes an area of the stopper through which reconstitution and withdrawal of the preparation can be made using a hypodermic needle and syringe. If use of a pipette is preferred, then fully remove the metal collar using, for example, forceps, taking care to avoid cuts by wearing appropriate gloves. Remove the stopper for access. Care should be taken to prevent loss of the contents.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the material

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Tel: +44 (0)1707 641000
Fax: +44 (0)1707 641050
Email: enquiries@nibsc.ac.uk
Web: <http://www.nibsc.ac.uk>

The vial should be stored at -20°C on receipt; each vial is intended to be used only once. The ampoule should be opened as directed in 6 and should then be reconstituted with 1.0ml of de-ionised, RNAase-free water immediately before use. The vials should be gently agitated over a minimum period of 20 minutes to fully dissolve the contents. The contents should be transferred to a screw-capped plastic vial and vortexed at full speed for 10 seconds before assaying.

8. STABILITY

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. Once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

NIBSC follows the policy of WHO with respect to its reference materials.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

WHO ECBS Report, 1999; Holmes H et al, 2001, J Virol. Meth. 92:141-150

10. ACKNOWLEDGEMENTS

Collaborative Study Group

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.ac.uk
WHO Biological Standards: <http://www.who.int/biologicals/en/>
Derivation of International Units:
<http://www.nibsc.ac.uk/products/faq.asp>
Ordering standards from NIBSC:
<http://www.nibsc.ac.uk/products/faq.asp>
NIBSC Terms & Conditions: <http://www.nibsc.ac.uk/terms.html>

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.ac.uk

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties	
Physical appearance: Freeze dried	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No

Appendix C Abbott RealTime HIV-1 Assay.

Abbott RealTime HIV-1

Abbott RealTime HIV-1

The Abbott RealTime HIV-1 assay is an *in vitro* reverse transcription-polymerase chain reaction (RT-PCR) assay for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) in human plasma from HIV-1 infected individuals. The Abbott RealTime HIV-1 assay is intended for use in conjunction with clinical presentation and other laboratory markers as an indicator of disease prognosis and for use as an aid in assessing viral response to anti-retroviral treatment as measured by changes in plasma HIV-1 RNA levels.

Abbott RealTime HIV-1 Correlation to Comparator Assay

Specimens from 142 HIV-1 infected patients were tested with the RealTime HIV-1 and a comparator assay per the NCCLS Guideline (NCCLS Document EP9-A2).

Abbott RealTime HIV-1 Performance*

Sensitivity	40 copies/ml for 1 ml input, 75 copies/ml for 0.5 ml input, 150 copies/ml for 0.2 ml input
Linear Range	40 copies/ml (1.6 log copies) to 10 million copies/ml (7.0 log copies/ml)
Precision	Inter-assay SD at 5.5 log copies/ml = 0.04, Inter-assay SD at 1.86 log copies/ml = 0.19
Specificity	$\geq 99.5\%$ [†]
HIV-1 Subtype Detection	Group M subtypes A–H, Group O and Group N
Standardization	To VQA and WHO standards
Internal Control	Added to lysis buffer during extraction and detected at all levels

* Performance data shown from internal verification. The Abbott RealTime HIV-1 assay is not intended to be used as a screening test for HIV-1 or as a diagnostic test to confirm the presence of HIV-1 infection.

[†] The specificity of the RealTime HIV-1 assay was evaluated by testing 189 HIV-1 seronegative plasma specimens, HIV-1 RNA was not detected, resulting in 100% (189/189) specificity in this representative study.

Abbott RealTime HIV-1 Detection of Subtypes and Groups

Group/Subtypes	n	RealTime Detected	Comparator 1 Detected	Comparator 2 Detected
M/Subtype A	10	10	10 (1)	10 (2)
M/Subtype B	10	10	10 (0)	10 (0)
M/Subtype C	10	10	10 (0)	10 (0)
M/Subtype D	10	10	10 (0)	10 (0)
M/Subtype AE	10	10	10 (0)	10 (0)
M/Subtype F	10	10	10 (0)	10 (0)
M/Subtype AG	10	10	10 (4)	10 (4)
M/Subtype G	10	10	10 (2)	10 (2)
Group O	8	8	0 (NA)	5 (5)

A total of 88 clinical specimens, ten of each Group M subtype (A, B, C, D, CRF01-AE, F, CRF02-AG, G) and eight of Group O, were tested with the RealTime HIV-1 assay and by two other HIV-1 quantitative assays referred to as Comparator 1 and Comparator 2. The numbers in parentheses are the number of specimens that had lower quantitation values by more than 1.00 log copies/ml when compared to RealTime HIV-1 assay.

Abbott RealTime HIV-1

Today's clinical molecular diagnostics laboratory must have confidence in the quality of the HIV-1 patient results. As a result of our real-time PCR development philosophy, Abbott RealTime HIV-1 is engineered to tolerate the genetic diversity of HIV-1 Subtypes.

HIV-1 diversity can be attributed to:

- Error-prone reverse transcriptase enzyme
- Recombination of subtypes
- Cross-species transmission

Accurate quantification is dependent upon a combination of:

- Primer design
- Probe design
- Cycling conditions

RealTime HIV-1 Probe Design: Probe Partially Double Stranded

In the absence of target, the probe hybridizes to the quencher oligonucleotide, preventing fluorescent signal generation.

In the presence of target, the probe prefers to hybridize with the target sequence, dissociating from the quencher oligonucleotide and allowing fluorescent detection.

Primer and Probe Design: RealTime HIV-1 Primers are Targeted to the Integrase Region of the Polymerase Gene

The integrase region of the polymerase gene is a conserved region of the HIV-1 genome.

Cycling Conditions: Low Temperature Read Cycles

The cycling conditions for Abbott RealTime HIV-1 encompass a low temperature read cycle. This read cycle allows the probe to tolerate mismatches more effectively than a probe required to bind during the extension phase.

The Abbott RealTime HIV-1 assay is designed to detect and quantify all HIV-1 Group M subtypes A–H, Group O, and Group N and provide sensitivity, specificity, and dynamic range to meet your laboratory needs.

Product	List Number	Configuration
Abbott RealTime HIV-1 Calibrator Kit	2G3170	12 Cal A, 12 Cal B, 4 Complete Calibration Sets
Abbott RealTime HIV-1 Control Kit	2G3180	8 Low Positive Control, 8 High Positive Control, 8 Negative Control
Abbott RealTime HIV-1 Amplification Reagent Kit	2G3190	96 Assays (4 packs x 24 assays)
Abbott RealTime HIV-1 Application CD-ROM	1L6801	1

Appendix D. COBAS AmpliPrep/COBAS TaqMan HIV-1

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test

FOR *IN VITRO* DIAGNOSTIC USE.

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test	HIMCAP	48 Tests	P/N: 03542998 190
COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent	PG WR	5.1 Liters	P/N: 03587797 190

INTENDED USE

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is an *in vitro* nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma using the COBAS® AmpliPrep Instrument for automated specimen processing and COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer for automated amplification and detection.

This test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progress for the clinical management of HIV-1 infected patients. The Test can be used to assess patient prognosis by measuring the baseline HIV-1 RNA level or to monitor the effects of antiretroviral therapy by measuring changes in EDTA plasma HIV-1 RNA levels during the course of antiretroviral treatment.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is not intended for use as a screening test for the presence of HIV-1 in blood or blood products or as a diagnostic test to confirm the presence of HIV-1 infection.

The test can quantitate HIV-1 RNA over the range of 48 - 10,000,000 copies/mL. One copy of HIV-1 RNA is equivalent to 1.7 ± 0.1 International Units (IU) based on the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656).

SUMMARY AND EXPLANATION OF THE TEST

Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS)¹⁻³. HIV infection can be transmitted by sexual contact, exposure to infected blood or blood products, or by an infected mother to the fetus⁴. Within three to six weeks of exposure to HIV, infected individuals generally develop a brief, acute syndrome characterized by flu-like symptoms and associated with high levels of viremia in the peripheral blood⁵⁻⁸. In most infected individuals this is followed by an HIV-specific immune response and a decline of plasma viremia, usually within four to six weeks of the onset of symptoms^{9,10}. After seroconversion, infected individuals typically enter a clinically stable, asymptomatic phase that can last for years¹¹⁻¹³. The asymptomatic period is characterized by persistent, low level plasma viremia¹⁴ and a gradual depletion of CD4⁺ T lymphocytes, leading to severe immunodeficiency, multiple opportunistic infections, malignancies and death¹⁵. Although virus levels in the peripheral blood are relatively low during the asymptomatic phase of the infection, virus replication and clearance appear to be dynamic processes in which high rates of virus production and infection of CD4⁺ cells are balanced by equally high rates of virus clearance, death of infected cells and replenishment of CD4⁺ cells, resulting in relatively stable levels of both plasma viremia and CD4⁺ cells¹⁶⁻¹⁸.

Quantitative measurements of HIV viremia in the peripheral blood have shown that higher virus levels may be correlated with increased risk of clinical progression of HIV disease, and that reductions in plasma virus levels may be associated with decreased risk of clinical progression¹⁹⁻²¹. Virus levels in the peripheral blood can be quantitated by measurement of the HIV p24 antigen in serum, by quantitative culture of HIV from plasma, or by direct measurement of viral RNA in plasma using nucleic acid amplification or signal amplification technologies²²⁻²⁶.

p24 antigen is the principal core protein of HIV and is found in serum either free or bound by anti-p24 antibody. Free p24 antigen can be measured with commercially available enzyme immunoassays (EIA), although the usefulness of p24 antigen as a marker of viral load is limited since the antigen is detectable in only 20% of asymptomatic patients and 40-50% of symptomatic patients. Procedures to dissociate antigen-antibody complexes improve the sensitivity of the p24 antigen tests, but the viral protein remains undetectable in most asymptomatic patients²².

Infectious HIV in plasma can be cultured by inoculation into activated peripheral blood mononuclear cells (PBMC) from normal donors. Quantitation is achieved by inoculating PBMC with serial dilutions of the plasma specimen. Quantitative culture has limited utility for monitoring virus levels in infected individuals since only a small fraction of virus particles is infectious *in vitro*. Infectious virus is often undetectable in asymptomatic individuals²².

HIV RNA in plasma can be quantitated by nucleic acid amplification technologies, such as the Polymerase Chain Reaction (PCR)²⁷⁻²⁹. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test uses PCR technology to achieve high sensitivity and dynamic range for the quantitative detection of HIV-1 RNA in EDTA anti-coagulated plasma.

PRINCIPLES OF THE PROCEDURE

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is a nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. Specimen preparation is automated using the COBAS® AmpliPrep Instrument with amplification and detection automated using the COBAS® TaqMan® Analyzer or the COBAS® TaqMan® 48 Analyzer.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is based on three major processes: (1) specimen preparation to isolate HIV-1 RNA; (2) reverse transcription of the target RNA to generate complementary DNA (cDNA), and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide probe specific to the target.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test permits automated specimen preparation followed by automated reverse transcription, PCR amplification and detection of HIV-1 target RNA and HIV-1 Quantitation Standard (QS) Armored RNA. The Master Mix reagent contains primers and probes specific for both HIV-1 RNA and HIV-1 QS RNA. The Master Mix has been developed to ensure equivalent quantitation of group M subtypes of HIV-1. The detection of amplified DNA is performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon.

The quantitation of HIV-1 viral RNA is performed using the HIV-1 QS. It compensates for effects of inhibition and controls the preparation and amplification processes, allowing a more accurate quantitation of HIV-1 RNA in each specimen. The HIV-1 QS is a non-infectious Armored RNA construct that contains HIV sequences with identical primer binding sites as the HIV-1 target RNA and a unique probe binding region that allows HIV-1 QS amplicon to be distinguished from HIV-1 target amplicon.

The HIV-1 QS is added to each specimen at a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification and detection steps of cleaved dual-labeled oligonucleotide detection probes. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer calculates the HIV-1 RNA concentration in the test specimens by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control.

Target Selection

Selection of the target RNA sequence for HIV-1 depends on identification of regions within the HIV-1 genome that show maximum sequence conservation among the various group M HIV-1 subtypes. Generic silica based specimen preparation is used to capture the HIV-1 RNA and HIV-1 QS RNA and defined oligonucleotides are used as primers in amplification of the HIV-1 RNA and HIV-1 QS RNA. A target-specific and a QS-specific dual-labeled oligonucleotide probe permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon. Accordingly, the appropriate selection of the primers and the dual-labeled oligonucleotide probe is critical to the ability of the test to amplify and detect the HIV-1 subtypes. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test uses reverse transcription and PCR amplification primers that define a sequence within the highly conserved region of the HIV-1 *gag* gene³². The *gag* region encodes the group-specific antigens or core structural proteins of the virion. The nucleotide sequence of the primers has been optimized to yield comparable amplification of group M subtypes of HIV-1.

Specimen Preparation

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test utilizes automated specimen preparation on the COBAS® AmpliPrep Instrument by a generic silica-based capture technique. The procedure processes 850 µL of plasma. The HIV-1 virus particles are lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HIV-1 RNA from RNases in plasma. Protease and a known number of HIV-1 QS Armored RNA molecules are introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HIV-1 RNA and HIV-1 QS RNA are bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins and other cellular impurities, are removed by washing the magnetic glass particles. After separating the magnetic glass particles and completing the washing steps, the adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HIV-1 RNA and HIV-1 QS RNA, is added to the amplification mixture and transferred to the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. The HIV-1 target RNA and the HIV-1 QS RNA are then reverse transcribed, amplified and simultaneously

detected by cleavage of a target-specific and a QS-specific dual-labeled oligonucleotide probe.

Reverse Transcription and PCR Amplification

The reverse transcription and PCR amplification reaction is performed with the thermostable recombinant enzyme *Thermus specie* DNA Polymerase (Z05). In the presence of manganese (Mn^{2+}) and under the appropriate buffer conditions, Z05 has both reverse transcriptase and DNA polymerase activity^{30,31}. This allows both reverse transcription and PCR amplification to occur together with real-time detection of the amplicon.

Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which both reverse transcription and PCR amplification occur. The reaction mixture is heated to allow a downstream primer to anneal specifically to the HIV-1 target RNA and to the HIV-1 QS RNA. In the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, Z05 polymerase extends the annealed primers forming a DNA strand complementary to the RNA target.

Target Amplification

Following reverse transcription of the HIV-1 target RNA and the HIV-1 QS RNA, the Thermal Cycler in the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer heats the reaction mixture to denature the RNA:cDNA hybrid and to expose the specific primer target sequences. As the mixture cools, the primers anneal to the target DNA. The thermostable *Thermus specie* Z05 DNA Polymerase (Z05) in the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, extends the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer automatically repeats this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. Amplification occurs only in the region of the HIV-1 genome between the primers; the entire HIV-1 genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine³³, but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also, any nonspecific product formed after initial activation of the Master Mix by manganese is destroyed by the AmpErase enzyme. The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme remains inactive for a prolonged period of time once exposed to temperatures above 55°C, i.e. throughout the thermal cycling steps, and therefore does not destroy target amplicon formed after PCR.

Detection of PCR Products in a COBAS® TaqMan® Test

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test utilizes real-time^{34,35} PCR technology. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HIV-1 and HIV-1 QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, the HIV-1 and HIV-1 QS probes are labeled with different fluorescent reporter dyes. When these probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5' → 3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HIV-1 RNA and HIV-1 QS RNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HIV-1 RNA and HIV-1 QS RNA. The PCR cycle where a growth curve starts exponential growth is related to the amount of starting material at the beginning of the PCR.

Fundamentals of COBAS® TaqMan® Test Quantitation

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is inherently quantitative over a very wide dynamic range since the monitoring of amplicon is performed during the exponential phase of amplification. The higher the HIV-1 titer of a specimen, the earlier the fluorescence of the reporter dye of the HIV-1 probe rises above the baseline fluorescence level (see Figure 1). Since the amount of HIV-1 QS RNA is constant between all specimens, the fluorescence of the reporter dye of the HIV-1 QS probe should appear at the same cycle for all specimens (see Figure 2). In specimens where the QS fluorescence is affected, the concentration is adjusted accordingly. The appearance of the specific fluorescent signals is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds a pre-determined threshold (the Assigned Fluorescence Level), and starts the exponential growth phase of this signal (see Figure 3). A higher Ct value indicates a lower titer of initial HIV-1 target material. A 2-fold increase in titer correlates with a decrease of 1 Ct for target HIV-1 RNA, while a 10-fold increase in titer correlates with a decrease of 3.3 Ct.

Figure 1 shows the target growth curves for a dilution series spanning a 5- $\log_{10}$ range. As the concentration of the virus increases, the growth curves shift to earlier cycles. Therefore, the leftmost growth curve corresponds to the highest viral titer level, whereas, the rightmost growth curve corresponds to the lowest viral titer level.

Figure 1
Target Growth Curves for a Dilution Series Spanning a 5- $\log_{10}$ Range

Figure 2 shows the Quantitation Standard growth curves for specimens from a viral dilution series that spans a 5- $\log_{10}$ range. The amount of Quantitation Standard added to each specimen is constant for each reaction. The Ct value of the Quantitation Standard is similar regardless of the target viral titer.

Figure 2
Quantitation Standard Growth Curves for Specimens from a Viral Dilution Series that Spans a 5- $\log_{10}$ Range

Figure 3 provides an example of how the fluorescence values at every cycle are normalized for each growth curve. The critical threshold value (Ct) is calculated where the fluorescence signal crosses the Assigned Fluorescence Level.

Figure 3
Normalization of Fluorescence Values at Every Cycle for Each Growth Curve

HIV-1 RNA Quantitation

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test quantitates HIV-1 viral RNA by utilizing a second target sequence (HIV-1 Quantitation Standard) that is added to each test specimen at a known concentration. The HIV-1 QS is a non-infectious Armored RNA construct, containing fragments of HIV-1 sequences with primer binding regions identical to those of the HIV-1 target sequence. The HIV-1 QS contains HIV-1 primer binding regions and generates an amplification product of the same length and base composition as the HIV-1 target RNA. The detection probe binding region of the HIV-1 QS has been modified to differentiate HIV-1 QS amplicon from HIV-1 target amplicon.

During the annealing phase of the PCR on the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer, the specimens are illuminated and excited by filtered light and filtered emission fluorescence data are collected for each specimen. The readings from each specimen are then corrected for instrumental fluctuations. These fluorescence readings are sent by the instrument to the AMPLILINK software and stored in a database. Pre-Checks are used to determine if the HIV-1 RNA and HIV-1 QS RNA data represent sets that are valid, and flags are generated when the data lie outside the preset limits. After all Pre-Checks are completed and passed, the fluorescence readings are processed to generate Ct values for the HIV-1 RNA and the HIV-1 QS RNA. The lot-specific calibration constants provided with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test are used to calculate the titer value for the specimens and controls based upon the HIV-1 RNA and HIV-1 QS RNA Ct values. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is standardized against the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656)³⁶. Titer results are reported in copies/mL (cp/mL). The conversion factor between reported HIV-1 RNA copies/mL and HIV-1 International Units/mL has been determined by Roche Molecular Systems, Inc. to be 0.6 cp/IU (1.7 IU/cp).

Appendix E. SOP for in-house HIV-1 Viral Load Quantification Real Time RT-PCR assay

Aim: To determine the viral load of the HIV-1 positive serum samples using Real time RT-PCR assay

Materials required:

- 1) QIAamp Viral RNA mini kit (catalog no.: 52906, Qiagen, Germany)
- 2) HotStarTaq Master mix kit (catalog no.: 203443, Qiagen, Germany)
- 3) pcR[®] 4-TOPO vector (catalog no.: 45-0030, Invitrogen, Carlsbad)
- 4) FINNZYMES Phire[™] Hot Star Taq DNA polymerase (catalog no.: F-120S, Finnzymes, Finland)
- 5) SuperScript[™] III RT/Platinum[®] Taq Mix (catalog no. 11732-20, Invitrogen, USA)

Steps required:

- 1) Extraction of HIV-1 RNA from plasma samples
- 2) Cloning of HIV external plasmid standards
- 3) Internal control plasmids
- 4) Real Time PCR set up and cycling conditions
- 5) Result analysis

1) Extraction of HIV-1 RNA from plasma samples

- i) Ultra-centrifuge 1mL patient plasma at 24,000xg for 60 minutes at 4⁰c.
- ii) Carefully discard 800µL of supernatant using 1ml micropipette.

- iii) Using 200µL of the reminding plasma containing the viral pellet, RNA extraction is to be carried out using QIAamp Viral RNA mini kit (catalog no.: 52906, Qiagen, Germany), following manufacturer's protocol.
- iv) Eluted purified RNA in 50µL of kit elution buffer and stored in -30⁰c freezer.

2) Cloning of HIV external plasmid standards

- i) External standard for the quantification of the viral RNA copies are synthesized from the RT-PCR amplification of HIV-1 positive patient sample (subtype AE).
- ii) Performed PCR with HotStarTaq Master mix kit (catalog no.: 203443, Qiagen, Germany) using detection primers gag183/187.
- iii) Purify the amplicons using QIAquick PCR purification kit (catalog no.: 28106, Qiagen, Germany).
- iv) Clone purified PCR product into the pcR[®] 4-TOPO vector (catalog no.: 45-0030, Invitrogen, Carlsbad), following manufacturer's protocol.
- v) Serially dilute the plasmid obtained in 0.1ng/µL of ssDNA and stored in -30⁰c for future assay.

3) Internal control molecule production

- i) Perform PCR using the primer gag187U/187L with an in vitro transcribed oligonucleotide molecule that incorporates a unique probes binding site different from the binding site of the HIV-1 target molecule. Amplification is carried out using FINNZYMES Phire[™] Hot Star Taq DNA polymerase (catalog no.: F-120S, Finnzymes, Finland).
- ii) Dilute IC molecule using 1ng/µL tRNA to 100copies/µL which will be added to each of the reaction well of the real time PCR assay.

4) Real time PCR assay

In the RNase-Free thin wall PCR tubes (0.5mL), add the following reagents per test/ per reaction using SuperScript™ III RT/Platinum® Taq Mix (catalog no. 11732-20, Invitrogen, USA)

Tube no.	Description	Volume (μL)	Final Concentration
Primer 1	HIV-1 upper primer, 50uM	0.15	0.3μM
Primer 2	HIV-1 lower primer, 50uM	0.15	0.3μM
Probe 1	HIV-1 Probe (FAM), 10uM	0.25	0.1μM
Probe 2	IC Probe (Texas Red), 10uM	0.25	0.1μM
IC	Internal control, 100copies/μL	1.00	100cp/μL
From Invitrogen Kit	SuperScript III RT Taq Polymerase mix	0.50	
	2x Reaction mix	12.50	
	H2O	0.20	
	RNA Sample	10.00	
	Total reaction vol.	25	

For each batch of the PCR runs, a standard set of external plasmid standards (with known concentration: 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 1 copies/μL) needs to be run in parallel to serve as a calibration curve to quantify the patient samples' viral load.

Thermal cycling conditions for real time PCR:

Step	Temp (⁰ c)	Stratagene (Mx3000P) Duration	Number of cycles	Step
1	55	30 min	1	Reverse transcription
2	95	2min 30sec	1	Initial activation
3	95	17 sec	48	Denaturation
	65	31 sec		Annealing
	68	32 sec		Extension

5) Data analysis with viral load quantification

Using Microsoft Excel, plot a standard curve with the Ct values of the external standards against the known copies number of the standards. Clinical samples' viral loads are determined with the extrapolation of the generated Ct value against the calibration curve. The final quantitative data of the samples viral load were calculated based on the initial plasma volume and the eluted volume during the extraction process.

Appendix F. IAS 2009 Poster Discussion (MOPDB101).

Poster Title: Accurate quantification of HIV-1 cDNA on a credit-card-sized microfluidic platform.

Accurate Quantification of HIV-1 on a credit-card-sized microfluidic platform

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Aim:

To develop a credit-card-sized microfluidic platform for accurate quantification of HIV-1.

Background

HIV-1 viral quantification is the most sensitive prognostic marker of treatment response and the only means of early postnatal diagnosis. Limitations in resources and laboratory facilities in resource-limited settings hamper widespread access. No reliable surrogate test in these settings is available.

We aim to develop a hand-held device that is able to accurately quantify for HIV-1 for use in these settings.

Methods

Our lab-on-chip (Figure 1) uses:

- Free droplet containing surface-functionalized magnetic particles to achieve a virtual micro-total analysis system (μ TAS) with (sub)microliter volumes (Figure 2).
- Magnetic particles as force mediators for actuating the droplet in a magnetic field and serving as solid support for performing biochemical processes.
- Novel miniaturized system for dual-channel detection.

We evaluated the performance of the lab-on-chip using a 1- μ L droplet containing 10^1 to 10^9 copies of HIV-1 cDNA with 10 copies of IC per reaction. The assay was performed using in-house designed primers with fluorescent probe targeting the HIV-1 *gag* region and internal control (IC) cDNA targets co-amplified by the primers for HIV-1 and detected by IC-specific fluorescent probes (Figure 3).

Figure 1 Photograph of the prototype. PCB 3 printed circuit board 3; R reference temperature sensor for calibrating the PCR chip; H1-H4 heaters/temperature sensors 1-4; G Teflon-coated glass substrate; P PCR chip; M magnet glued to a DI rotating disc; D miniaturized dual-channel fluorescence detector. Scale bar, 10 mm (white line on PCB 3).

Figure 2 Droplet arrangement. PCB printed circuit board; G teflonized glass; M magnet; T thermocycler; Sa raw sample solution; W1, W2 washing solutions; R (blue) RT-PCR mixture covered by mineral oil. Scale bar, 10 mm.

Results

Target cycle-threshold results ranged on a log-linear scale from Ct 37.2 (10^1 c/rxn) to 8.9 (10^9 c/rxn) for the hand-held device ($R^2=1.00$) and Ct 36.9 (10^1 c/rxn) to 8.7 (10^9 c/rxn) ($R^2=1.00$). The IC was consistently detected except at the highest concentrations, thereby reliably excluding reaction inhibition (figure 3). The correlation between the two devices was excellent (graph 1).

Figure 3 Detection of 10-fold serial dilutions of HIV-1 cDNA with 10 copies of internal control by our device using a common pair of primers, targeting the *gag* region of HIV-1. FAM- and TexRd- labelled TaqMan probes were used to specifically detect HIV target and internal control respectively.

Graph 1. Correlation between hand-held device and Stratagene Mx3000P QPCR thermocycler

Graph 1 Correlation of HIV-1 cDNA copies ($\log_{10}$) detected between hand-held device and Stratagene Mx3000P QPCR thermocycler. Equation of fitted regression line: $y = 0.9992x + 0.0041$, coefficient of determination, $r^2 = 0.9973$

Conclusion

We have demonstrated the ability of our hand-held device, to accurately quantify HIV-1 cDNA using duplex real-time PCR, comparable to a conventional bench-top QR T-PCR thermocycler.

We are currently incorporating direct one-step blood sample preparation to actualize a field-ready device.

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Appendix G. Viral load result for 329 HIV patient samples.

Objective: Comparison of viral load quantitation of HIV patient samples between in house and ABBOT commercial assay kit.

Group 1: ABBOTT Automatic extraction- ABBOTT real time HIV-1 kit

Group 2: QIAgen manual extraction-in house assay (adjusted with 2.49 factor)

No.	TTSH Study No.	Group 1			Group 2		Mean	Differences/ (IH-Commerical)
		Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)		
1	MOVL005	Detected <40	-	-	-	-	-	-
2	MOVL006	1.69E+04	4.227	2.28E+04	5.69E+04	4.755	4.491	0.527
3	MOVL007	9.84E+03	3.993	1.20E+04	2.98E+04	4.475	4.234	0.482
4	MOVL008	2.09E+04	4.320	1.34E+03	3.34E+03	3.524	3.922	-0.796
5	MOVL009	7.50E+01	1.875	3.43E+00	8.53E+00	0.931	1.403	-0.944
6	MOVL014	5.37E+04	4.730	6.25E+03	1.56E+04	4.192	4.461	-0.538
7	MOVL015	1.27E+04	4.102	1.65E+03	4.10E+03	3.613	3.857	-0.490
8	MOVL016	2.76E+03	3.441	2.72E+02	6.77E+02	2.831	3.136	-0.610
9	MOVL019	4.27E+04	4.630	6.05E+03	1.51E+04	4.178	4.404	-0.452
10	MOVL020	1.50E+05	5.176	6.83E+03	1.70E+04	4.231	4.704	-0.946
11	MOVL022	1.86E+04	4.269	1.61E+03	4.02E+03	3.604	3.937	-0.665
12	MOVL024	8.29E+04	4.918	1.05E+04	2.63E+04	4.419	4.669	-0.499
13	MOVL031	Detected <40	-	-	-	-	-	-
14	MOVL035	5.29E+03	3.723	8.69E+02	2.16E+03	3.335	3.529	-0.388
15	MOVL036	4.04E+02	2.606	3.68E+01	9.18E+01	1.963	2.285	-0.644
16	MOVL037	1.57E+04	4.196	8.49E+03	2.11E+04	4.325	4.261	0.129
17	MOVL038	Detected <40	-	-	-	-	-	-
18	MOVL042	9.22E+02	2.965	2.01E+02	5.01E+02	2.700	2.832	-0.265
19	MOVL043	1.41E+02	2.149	3.64E+01	9.06E+01	1.957	2.053	-0.192
20	MOVL047	1.45E+04	4.160	6.33E+03	1.58E+04	4.198	4.179	0.038
21	MOVL048	1.20E+04	4.078	4.60E+03	1.14E+04	4.059	4.068	-0.019
22	MOVL049	5.38E+02	2.731	3.00E+02	7.46E+02	2.873	2.802	0.142
23	MOVL050	2.08E+02	2.318	3.61E+02	8.98E+02	2.953	2.636	0.635
24	MOVL053	Detected <40	-	-	-	-	-	-
25	MOVL054	2.00E+03	3.301	2.68E+03	6.68E+03	3.825	3.563	0.523
26	MOVL055	Detected <40	-	-	-	-	-	-
27	MOVL057	2.25E+02	2.352	2.85E+01	7.10E+01	1.851	2.102	-0.501
28	MOVL058	5.53E+02	2.743	4.48E+02	1.12E+03	3.048	2.895	0.305
29	MOVL060	Detected <40	-	4.59E+00	1.14E+01	1.058	-	-
30	MOVL061	9.00E+03	3.954	2.15E+04	5.35E+04	4.728	4.341	0.774
31	MOVL063	3.95E+04	4.597	1.45E+05	3.60E+05	5.556	5.077	0.960
32	MOVL064	Detected <40	-	4.79E+01	1.19E+02	2.077	-	-
33	MOVL066	4.80E+01	1.681	-	-	-	-	-
34	MOVL067	3.08E+05	5.489	7.55E+04	1.88E+05	5.274	5.381	-0.215
35	MOVL071	Detected <40	-	1.89E+00	4.71E+00	0.673	-	-
36	MOVL072	Detected <40	-	-	-	-	-	-
37	MOVL075	Detected <40	-	-	-	-	-	-
38	MOVL076	Detected <40	-	2.21E+01	5.51E+01	1.741	-	-
39	MOVL078	Detected <40	-	-	-	-	-	-
40	MOVL081	1.21E+04	4.084	3.48E+03	8.66E+03	3.938	4.011	-0.146
41	MOVL084	1.36E+02	2.134	5.99E+01	1.49E+02	2.174	2.154	0.040
42	MOVL085	Detected <40	-	-	-	-	-	-
43	MOVL087	Detected <40	-	2.66E+01	6.63E+01	1.821	-	-
44	MOVL088	4.89E+05	5.689	1.40E+05	3.49E+05	5.543	5.616	-0.146
45	MOVL089	1.00E+05	5.000	6.42E+04	1.60E+05	5.204	5.102	0.204
46	MOVL090	1.48E+02	2.170	3.05E+01	7.60E+01	1.881	2.025	-0.290
47	MOVL094	3.49E+05	5.543	1.67E+05	4.15E+05	5.618	5.581	0.075
48	MOVL096	3.20E+03	3.505	2.87E+04	7.15E+04	4.855	4.180	1.350
49	MOVL097	2.09E+03	3.320	6.05E+02	1.51E+03	3.178	-	-
50	MOVL100	Detected <40	-	7.84E+00	1.95E+01	1.290	-	-

No.	TTSH Study No.	Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)	Mean	Differences/ (IH- Commerical)
51	MOVL102	Detected <40	-	-	-	-	-	-
52	MOVL104	Detected <40	-	-	-	-	-	-
53	MOVL105	Detected <40	-	-	-	-	-	-
54	MOVL108	1.11E+04	4.044	6.87E+03	1.71E+04	4.233	4.139	0.189
55	MOVL109	Detected <40	-	-	-	-	-	-
56	MOVL113	1.21E+02	2.083	-	-	-	-	-
57	MOVL115	1.07E+02	2.029	1.08E+01	2.69E+01	1.430	1.729	-0.600
58	MOVL118	Detected <40	-	-	-	-	-	-
59	MOVL119	1.41E+04	4.150	1.02E+04	2.54E+04	4.405	4.278	0.256
60	MOVL120	1.05E+04	4.020	4.26E+03	1.06E+04	4.026	4.023	0.006
61	MOVL121	2.13E+04	4.328	1.10E+04	2.74E+04	4.438	4.383	0.110
62	MOVL125	5.69E+02	2.755	1.96E+02	4.88E+02	2.688	2.722	-0.067
63	MOVL127	2.07E+04	4.315	7.18E+03	1.79E+04	4.253	4.284	-0.063
64	MOVL130	9.10E+01	1.959	4.47E+01	1.11E+02	2.047	2.003	0.088
65	MOVL131	4.50E+01	1.653	7.99E+00	1.99E+01	1.299	1.476	-0.355
66	MOVL132	1.55E+03	3.189	8.47E+02	2.11E+03	3.324	3.257	0.135
67	MOVL133	8.77E+03	3.943	3.67E+03	9.13E+03	3.960	3.952	0.018
68	MOVL134	9.27E+04	4.967	1.31E+04	3.25E+04	4.512	4.740	-0.455
69	MOVL135	4.99E+02	2.698	7.49E+01	1.86E+02	2.270	2.484	-0.428
70	MOVL136	7.54E+03	3.877	4.54E+03	1.13E+04	4.053	3.965	0.176
71	MOVL141	4.40E+01	1.643	-	-	-	-	-
72	MOVL142	9.70E+01	1.987	9.39E+01	2.34E+02	2.369	2.178	0.382
73	MOVL143	5.07E+03	3.705	1.79E+03	4.46E+03	3.649	3.677	-0.055
74	MOVL145	Detected <40	-	-	-	-	-	-
75	MOVL147	9.02E+04	4.955	3.57E+04	8.89E+04	4.949	4.952	-0.007
76	MOVL148	3.10E+05	5.491	1.11E+05	2.77E+05	5.443	5.467	-0.048
77	MOVL150	7.82E+05	5.893	1.40E+05	3.48E+05	5.541	5.717	-0.352
78	MOVL151	Detected <40	-	-	-	-	-	-
79	MOVL154	1.01E+05	5.003	1.75E+04	4.37E+04	4.640	4.822	-0.363
80	MOVL156	Detected <40	-	4.89E+00	1.22E+01	1.086	-	-
81	MOVL159	Detected <40	-	-	-	-	-	-
82	MOVL160	5.87E+04	4.768	3.48E+04	8.67E+04	4.938	4.853	0.170
83	MOVL161	3.50E+03	3.544	1.32E+03	3.28E+03	3.515	3.530	-0.029
84	MOVL167	1.42E+04	4.152	6.79E+03	1.69E+04	4.228	4.190	0.076
85	MOVL169	4.40E+01	1.643	1.55E+01	3.87E+01	1.588	1.616	-0.056
86	MOVL170	Detected <40	-	-	-	-	-	-
87	MOVL171	Detected <40	-	7.36E+00	1.83E+01	1.263	-	-
88	MOVL173	Detected <40	-	-	-	-	-	-
89	MOVL177	1.99E+05	5.298	1.19E+05	2.95E+05	5.470	5.384	0.172
90	MOVL178	Detected <40	-	4.31E+01	1.07E+02	2.030	-	-
91	MOVL181	6.78E+04	4.831	2.16E+04	5.37E+04	4.730	4.781	-0.101
92	MOVL183	8.17E+03	3.912	7.94E+03	1.98E+04	4.296	4.104	0.384
93	MOVL184	4.07E+03	3.610	1.99E+03	4.96E+03	3.696	3.653	0.086
94	MOVL185	Detected <40	-	-	-	-	-	-
95	MOVL187	2.38E+02	2.377	1.77E+02	4.41E+02	2.644	2.511	0.268
96	MOVL193	1.76E+05	5.244	1.90E+04	4.74E+04	4.676	4.960	-0.569
97	MOVL194	Detected <40	-	-	-	-	-	-
98	MOVL200	7.10E+01	1.851	1.82E+02	4.52E+02	2.655	2.253	0.804
99	MOVL201	2.67E+03	3.427	6.30E+03	1.57E+04	4.195	3.811	0.768
100	MOVL202	Detected <40	-	1.41E+00	3.51E+00	0.545	-	-

No.	TTSH Study No.	Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)	Mean	Differences/ (IH-Commerical)
101	MOVL204	Detected <40	-	5.18E+00	1.29E+01	1.110	-	-
102	MOVL211	1.38E+05	5.140	1.90E+05	4.73E+05	5.675	5.407	0.535
103	MOVL215	1.23E+02	2.090	2.89E+02	7.20E+02	2.857	2.474	0.768
104	MOVL216	9.10E+01	1.959	1.56E+01	3.90E+01	1.591	1.775	-0.368
105	MOVL219	5.32E+02	2.726	6.51E+02	1.62E+03	3.210	2.968	0.484
106	MOVL221	6.95E+05	5.842	2.27E+05	5.64E+05	5.751	5.797	-0.090
107	MOVL222	1.92E+03	3.283	6.27E+02	1.56E+03	3.193	3.238	-0.090
108	MOVL224	6.49E+04	4.813	1.77E+04	4.39E+04	4.643	4.728	-0.170
109	MOVL226	7.39E+03	3.869	4.66E+03	1.16E+04	4.064	3.966	0.196
110	MOVL230	Detected <40	-	-	-	-	-	-
111	MOVL231	1.07E+02	2.029	2.37E+01	5.90E+01	1.771	1.900	-0.259
112	MOVL232	Detected <40	-	6.49E+00	1.62E+01	1.208	-	-
113	MOVL233	Not detected	-	-	-	-	-	-
114	MOVL234	2.78E+04	4.444	1.81E+04	4.51E+04	4.654	4.549	0.210
115	MOVL235	Not detected	-	-	-	-	-	-
116	MOVL236	1.35E+04	4.131	6.18E+03	1.54E+04	4.187	4.159	0.056
117	MOVL237	Not detected	-	-	-	-	-	-
118	MOVL238	Not detected	-	-	-	-	-	-
119	MOVL239	Not detected	-	-	-	-	-	-
120	MOVL240	Not detected	-	-	-	-	-	-
121	MOVL241	9.50E+01	1.978	4.83E+01	1.20E+02	2.080	2.029	0.103
122	MOVL242	Not detected	-	1.36E+01	3.38E+01	1.529	-	-
123	MOVL243	7.10E+01	1.851	1.88E+00	4.69E+00	0.671	1.261	-1.181
124	MOVL244	6.20E+01	1.792	9.81E+00	2.44E+01	1.388	1.590	-0.405
125	MOVL245	2.70E+05	5.431	7.79E+04	1.94E+05	5.288	5.359	-0.143
126	MOVL246	Not detected	-	-	-	-	-	-
127	MOVL247	Not detected	-	-	-	-	-	-
128	MOVL248	Not detected	-	-	-	-	-	-
129	MOVL249	4.05E+04	4.607	1.34E+04	3.35E+04	4.524	4.566	-0.083
130	MOVL250	Not detected	-	-	-	-	-	-
131	MOVL251	Not detected	-	-	-	-	-	-
132	MOVL252	Not detected	-	9.74E+00	2.43E+01	1.385	-	-
133	MOVL252	Not detected	-	-	-	-	-	-
134	MOVL253	7.60E+01	1.881	3.18E+01	7.91E+01	1.898	1.890	0.017
135	MOVL254	Not detected	-	-	-	-	-	-
136	MOVL255	1.72E+03	3.235	8.58E+02	2.14E+03	3.330	3.282	0.094
137	MOVL256	3.71E+04	4.569	6.42E+04	1.60E+05	5.203	4.886	0.634
138	MOVL257	Detected <40	-	-	-	-	-	-
139	MOVL258	Not detected	-	-	-	-	-	-
140	MOVL259	6.80E+05	5.832	5.15E+04	1.28E+05	5.108	5.470	-0.724
141	MOVL260	Not detected	-	-	-	-	-	-
142	MOVL261	Not detected	-	-	-	-	-	-
143	MOVL262	Not detected	-	-	-	-	-	-
144	MOVL263	Not detected	-	-	-	-	-	-
145	MOVL264	4.48E+04	4.651	1.00E+04	2.49E+04	4.397	4.524	-0.255
146	MOVL265	4.43E+04	4.646	2.74E+03	6.83E+03	3.835	4.240	-0.811
147	MOVL267	Detected <40	-	-	-	-	-	-
148	MOVL268	Not detected	-	-	-	-	-	-
149	7007	1.12E+02	2.049	4.31E+02	1.07E+03	3.031	2.342	0.585
150	7011	6.20E+01	1.792	1.58E+01	3.94E+01	1.595	1.496	-0.593

No.	TTSH Study No.	Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)	Mean	Differences/ (IH- Commerical)
151	7013	9.70E+01	1.987	3.10E+01	7.72E+01	1.887	1.739	-0.496
152	7019	4.10E+01	1.613	1.72E+01	4.27E+01	1.631	1.424	-0.378
153	7031	2.47E+02	2.393	2.60E+02	6.48E+02	2.812	2.404	0.023
154	7039	1.42E+05	5.151	6.45E+04	1.61E+05	5.206	4.981	-0.341
155	7041	8.90E+01	1.949	2.20E+01	5.48E+01	1.739	1.646	-0.607
156	7055	1.51E+03	3.178	1.31E+03	3.27E+03	3.514	3.148	-0.060
157	7056	4.79E+04	4.680	1.14E+04	2.83E+04	4.452	4.368	-0.625
158	7058	5.38E+02	2.731	3.73E+02	9.30E+02	2.968	2.651	-0.159
159	7061	3.62E+02	2.559	3.47E+02	8.63E+02	2.936	2.549	-0.019
160	7064	4.57E+04	4.660	5.92E+04	1.47E+05	5.168	4.716	0.112
161	7065	4.10E+01	1.613	4.20E+01	1.05E+02	2.020	1.618	0.011
162	7068	4.00E+03	3.602	2.46E+03	6.13E+03	3.787	3.496	-0.211
163	7075	2.09E+04	4.320	1.10E+04	2.75E+04	4.439	4.181	-0.278
164	7076	3.97E+03	3.599	1.94E+04	4.83E+04	4.684	3.943	0.689
165	7077	1.93E+04	4.287	7.00E+04	1.74E+05	5.241	4.566	0.558
166	7087	9.84E+03	3.993	2.30E+04	5.73E+04	4.758	4.177	0.369
167	7092	1.15E+04	4.059	4.20E+04	1.05E+05	5.019	4.341	0.564
168	7095	3.88E+04	4.589	2.71E+05	6.76E+05	5.830	5.011	0.844
169	7100	7.53E+04	4.877	2.18E+05	5.43E+05	5.735	5.108	0.462
170	7105	1.93E+05	5.286	1.71E+06	4.26E+06	6.630	5.760	0.948
171	7109	1.42E+04	4.153	3.12E+04	7.76E+04	4.890	4.324	0.340
172	7110	1.37E+04	4.135	1.28E+04	3.19E+04	4.504	4.122	-0.027
173	7113	5.08E+02	2.706	2.07E+03	5.16E+03	3.712	3.011	0.610
174	7115	2.81E+02	2.449	1.11E+03	2.77E+03	3.443	2.748	0.598
175	7124	1.57E+04	4.195	4.43E+04	1.10E+05	5.043	4.421	0.452
176	7125	1.83E+03	3.263	6.69E+03	1.67E+04	4.222	3.544	0.563
177	7127	1.19E+04	4.075	2.52E+04	6.27E+04	4.797	4.238	0.326
178	7134	3.16E+04	4.499	1.01E+05	2.52E+05	5.402	4.753	0.507

Objective: Comparison of viral load quantitation of HIV patient samples between in house and ABBOT commercial assay kit.

Group 1: ROCHE Automatic extraction- ROCHE real time HIV-1 kit

Group 2: QIAgen manual extraction-in house assay (adjusted with 2.49 factor)

No.	TTSH Study No.	Group 1				Group 2		
		Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)	Mean	Differences/ (IH-Commercial)
1	FREE 001	5.57E+04	4.746	6.41E+03	1.60E+04	4.203	4.475	-0.543
2	FREE 002	3.79E+05	5.579	4.50E+04	1.12E+05	5.049	5.314	-0.530
3	FREE 003	8.21E+03	3.914	1.26E+04	3.13E+04	4.495	4.205	0.581
4	FREE 004	2.05E+02	2.312	2.17E+02	5.41E+02	2.733	2.523	0.422
5	FREE 005	1.72E+05	5.236	1.17E+05	2.92E+05	5.465	5.350	0.230
6	FREE 006	2.19E+04	4.340	5.37E+04	1.34E+05	5.127	4.733	0.786
7	FREE 007	4.79E+04	4.680	2.02E+03	5.03E+03	3.702	4.191	-0.978
8	FREE 008	1.06E+05	5.025	2.09E+04	5.20E+04	4.716	4.871	-0.310
9	FREE 009	1.91E+05	5.281	1.51E+04	3.76E+04	4.575	4.928	-0.706
10	FREE 010	3.84E+03	3.584	6.91E+02	1.72E+03	3.236	3.410	-0.349
11	FREE 011	1.23E+05	5.090	9.69E+03	2.41E+04	4.382	4.736	-0.708
12	FREE 012	2.93E+05	5.467	4.91E+04	1.22E+05	5.088	5.277	-0.379
13	FREE 013	7.71E+04	4.887	1.86E+03	4.64E+03	3.666	4.277	-1.221
14	FREE 014	9.93E+03	3.997	2.64E+04	6.57E+04	4.818	4.407	0.821
15	FREE 015	1.61E+05	5.207	1.93E+04	4.82E+04	4.683	4.945	-0.524
16	FREE 016	7.18E+02	2.856	5.51E+04	1.37E+05	5.137	3.997	2.281
17	FREE 017	1.14E+02	2.057	2.95E+01	7.34E+01	1.866	1.961	-0.191
18	FREE 018	3.14E+02	2.497	2.52E+02	6.28E+02	2.798	2.647	0.301
19	FREE 019	7.96E+04	4.901	2.38E+04	5.94E+04	4.774	4.837	-0.127
20	FREE 020	6.02E+04	4.780	2.07E+04	5.16E+04	4.713	4.746	-0.067
21	FREE 021	4.75E+04	4.677	2.13E+04	5.30E+04	4.724	4.700	0.047
22	FREE 022	2.92E+04	4.465	8.64E+03	2.15E+04	4.333	4.399	-0.133
23	FREE 023	< 40	-	-	-	-	-	-
24	FREE 024	1.12E+05	5.049	1.58E+04	3.93E+04	4.594	4.822	-0.455
25	FREE 025	< 40	-	-	-	-	-	-
26	FREE 026	1.75E+05	5.243	1.10E+04	2.74E+04	4.437	4.840	-0.806
27	FREE 027	3.94E+06	6.595	6.46E+05	1.61E+06	6.206	6.401	-0.389
28	FREE 028	< 40	-	-	-	-	-	-
29	FREE 029	1.61E+05	5.207	1.15E+04	2.86E+04	4.457	4.832	-0.750
30	FREE 030	< 40	-	5.37E+00	1.34E+01	1.126	-	-
31	FREE 031	5.97E+04	4.776	1.53E+04	3.81E+04	4.581	4.678	-0.195
32	FREE 032	3.10E+04	4.491	1.38E+03	3.44E+03	3.537	4.014	-0.955
33	FREE 033	2.36E+04	4.373	7.52E+03	1.87E+04	4.272	4.323	-0.101
34	FREE 034	2.91E+05	5.464	5.79E+04	1.44E+05	5.159	5.312	-0.305
35	FREE 035	1.70E+03	3.230	6.13E+03	1.53E+04	4.184	3.707	0.953
36	FREE 036	1.10E+05	5.041	1.11E+04	2.77E+04	4.443	4.742	-0.598
37	FREE 037	2.09E+04	4.320	2.81E+03	7.00E+03	3.845	4.083	-0.475
38	FREE 038	1.10E+05	5.041	2.01E+04	5.00E+04	4.699	4.870	-0.342
39	FREE 039	1.43E+03	3.155	2.88E+03	7.18E+03	3.856	3.506	0.701
40	FREE 040	6.97E+04	4.843	1.70E+04	4.24E+04	4.628	4.735	-0.216
41	FREE 041	7.33E+03	3.865	4.47E+03	1.11E+04	4.046	3.956	0.181
42	FREE 042	2.11E+04	4.324	4.01E+03	9.99E+03	3.999	4.162	-0.325
43	FREE 043	1.32E+04	4.121	6.17E+02	1.54E+03	3.187	3.654	-0.934
44	FREE 044	3.82E+03	3.582	5.01E+02	1.25E+03	3.096	3.339	-0.486
45	FREE 045	< 40	-	-	-	-	-	-
46	FREE 046	4.38E+03	3.641	2.29E+03	5.71E+03	3.757	3.699	0.115
47	FREE 047	5.07E+03	3.705	1.78E+02	4.43E+02	2.647	3.176	-1.058
48	FREE 048	1.28E+06	6.107	6.83E+04	1.70E+05	5.231	5.669	-0.876
49	FREE 049	< 40	-	-	-	-	-	-
50	FREE 050	1.42E+03	3.152	1.08E+03	2.69E+03	3.429	3.291	0.277

No.	TTSH Study No.	Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)	Mean	Differences/ (IH- Commerical)
51	FREE 051	< 40	-	2.54E+00	6.32E+00	0.801	-	-
52	FREE 052	1.09E+05	5.037	2.03E+04	5.07E+04	4.705	4.871	-0.333
53	FREE 053	1.21E+05	5.083	2.41E+04	6.01E+04	4.779	4.931	-0.304
54	FREE 054	< 40	-	-	-	-	-	-
55	FREE 055	1.37E+04	4.137	1.18E+03	2.94E+03	3.468	3.802	-0.669
56	FREE 056	4.86E+02	2.687	7.66E+01	1.91E+02	2.280	2.483	-0.406
57	FREE 057	7.54E+04	4.877	7.67E+03	1.91E+04	4.281	4.579	-0.597
58	FREE 058	6.88E+04	4.838	3.82E+03	9.52E+03	3.979	4.408	-0.859
59	FREE 059	3.01E+04	4.479	4.40E+03	1.10E+04	4.040	4.259	-0.439
60	FREE 060	5.00E+04	4.699	4.90E+03	1.22E+04	4.087	4.393	-0.612
61	FREE 061	3.98E+05	5.600	4.01E+04	9.98E+04	4.999	5.299	-0.601
62	FREE 062	1.24E+02	2.093	7.10E+00	1.77E+01	1.247	1.670	-0.846
63	FREE 063	1.14E+04	4.057	1.82E+03	4.54E+03	3.657	3.857	-0.400
64	FREE 065	1.27E+05	5.104	1.59E+04	3.95E+04	4.597	4.850	-0.507
65	FREE 066	1.44E+04	4.158	1.56E+03	3.89E+03	3.590	3.874	-0.568
66	FREE 067	1.88E+05	5.274	1.25E+04	3.12E+04	4.494	4.884	-0.780
67	FREE 068	5.27E+04	4.722	1.23E+04	3.06E+04	4.486	4.604	-0.236
68	FREE 069	6.40E+05	5.806	1.37E+05	3.40E+05	5.531	5.669	-0.275
69	FREE 070	2.23E+05	5.348	1.00E+05	2.50E+05	5.398	5.373	0.050
70	FREE 071	< 40	-	-	-	-	-	-
71	FREE 072	1.65E+03	3.217	2.35E+02	5.84E+02	2.767	2.992	-0.451
72	FREE 073	6.07E+04	4.783	1.84E+04	4.58E+04	4.661	4.722	-0.123
73	FREE 076	6.77E+02	2.831	9.24E+01	2.30E+02	2.362	2.596	-0.469
74	FREE 077	3.51E+05	5.545	4.47E+04	1.11E+05	5.046	5.296	-0.499
75	FREE 078	5.92E+03	3.772	3.18E+03	7.91E+03	3.898	3.835	0.126
76	FREE 079	< 40	-	-	-	-	-	-
77	FREE 080	< 40	-	-	-	-	-	-
78	FREE 081	1.36E+02	2.134	-	-	-	-	-
79	FREE 082	1.17E+05	5.068	8.33E+04	2.07E+05	5.317	5.193	0.249
80	FREE 083	4.05E+04	4.607	1.02E+04	2.53E+04	4.403	4.505	-0.205
81	FREE 084	2.24E+03	3.350	1.32E+03	3.28E+03	3.515	3.433	0.165
82	FREE 085	1.91E+05	5.281	1.31E+05	3.26E+05	5.513	5.397	0.232
83	FREE 086	< 40	-	-	-	-	-	-
84	FREE 087	5.14E+04	4.711	2.87E+04	7.15E+04	4.855	4.783	0.144
85	FREE 088	< 40	-	-	-	-	-	-
86	FREE 089	1.70E+03	3.230	1.87E+03	4.66E+03	3.669	3.450	0.438
87	FREE 090	< 40	-	-	-	-	-	-
88	FREE 091	1.39E+05	5.143	1.20E+05	2.99E+05	5.476	5.309	0.333
89	FREE 092	2.10E+04	4.322	5.74E+03	1.43E+04	4.155	4.239	-0.167
90	FREE 093	1.70E+04	4.230	1.99E+04	4.96E+04	4.696	4.463	0.465
91	FREE 094	6.04E+04	4.781	2.51E+04	6.24E+04	4.795	4.788	0.014
92	FREE 095	1.72E+03	3.236	1.56E+02	3.89E+02	2.590	2.913	-0.645
93	FREE 096	< 40	-	6.14E+01	1.53E+02	2.184	-	-
94	FREE 097	1.61E+04	4.207	2.52E+04	6.28E+04	4.798	4.502	0.591
95	FREE 098	< 40	-	-	-	-	-	-
96	FREE 099	5.30E+02	2.724	7.13E+03	1.78E+04	4.250	3.487	1.525
97	FREE 100	1.42E+04	4.152	6.30E+03	1.57E+04	4.196	4.174	0.043
98	FREE 101	2.94E+04	4.468	2.02E+04	5.03E+04	4.701	4.585	0.233
99	FREE 102	1.53E+05	5.185	2.68E+04	6.68E+04	4.825	5.005	-0.360
100	FREE 103	8.87E+04	4.948	5.14E+04	1.28E+05	5.107	5.028	0.159

No.	TTSH Study No.	Commerical Viral load (copies/mL)	Commerical Log10(copies/mL)	IH viral load (copies/mL)	Adjusted IH viral load (copies/mL)	IH viral load Log10(copies/mL)	Mean	Differences/ (IH- Commerical)
101	FREE 104	< 40	-	4.05E+00	1.01E+01	1.004	-	-
102	FREE 105	2.50E+05	5.398	3.44E+04	8.56E+04	4.932	5.165	-0.465
103	FREE 106	4.49E+04	4.652	2.63E+04	6.56E+04	4.817	4.735	0.165
104	FREE 107	1.19E+03	3.076	6.70E+02	1.67E+03	3.222	3.149	0.147
105	FREE 108	< 40	-	-	-	-	-	-
106	FREE 109	2.06E+03	3.314	1.09E+03	2.72E+03	3.435	3.374	0.121
107	FREE 110	< 40	-	-	-	-	-	-
108	FREE 111	2.88E+04	4.459	2.63E+04	6.56E+04	4.817	4.638	0.357
109	FREE 112	58	1.763	-	-	-	-	-
110	FREE 113	7.04E+04	4.848	2.31E+04	5.76E+04	4.760	4.804	-0.087
111	FREE 114	< 40	-	-	-	-	-	-
112	FREE 116	5.49E+05	5.740	2.60E+04	6.48E+04	4.811	5.276	-0.928
113	FREE 117	2.20E+05	5.342	4.71E+04	1.17E+05	5.069	5.206	-0.273
114	FREE 118	< 40	-	-	-	-	-	-
115	FREE 119	3.36E+04	4.526	1.86E+04	4.64E+04	4.666	4.596	0.140
116	FREE 120	5.25E+05	5.720	1.29E+05	3.21E+05	5.506	5.613	-0.214
117	FREE 121	4.89E+04	4.689	2.23E+04	5.56E+04	4.745	4.717	0.056
118	FREE 122	3.87E+05	5.588	8.73E+04	2.17E+05	5.337	5.462	-0.251
119	FREE 123	1.18E+05	5.072	1.00E+05	2.49E+05	5.396	5.234	0.324
120	FREE 124	3.41E+04	4.533	1.76E+04	4.37E+04	4.641	4.587	0.108
121	FREE 125	4.09E+04	4.612	2.06E+05	5.12E+05	5.709	5.160	1.098
122	FREE 126	3.22E+04	4.508	1.14E+04	2.85E+04	4.455	4.481	-0.053
123	FREE 127	< 40	-	-	-	-	-	-
124	FREE 128	4.40E+04	4.643	1.43E+04	3.55E+04	4.551	4.597	-0.093
125	FREE 129	5.58E+03	3.747	1.22E+03	3.04E+03	3.482	3.614	-0.264
126	FREE 130	2.39E+04	4.378	1.89E+05	4.71E+05	5.673	5.026	1.294
127	FREE 131	5.23E+04	4.719	9.99E+03	2.49E+04	4.396	4.557	-0.323
128	FREE 133	6.60E+01	1.820	9.94E+00	2.47E+01	1.394	1.607	-0.426
129	FREE 134	< 40	-	-	-	-	-	-
130	FREE 135	1.91E+05	5.281	1.03E+05	2.56E+05	5.408	5.344	0.127
131	FREE 136	8.87E+03	3.948	3.83E+04	9.53E+04	4.979	4.464	1.031
132	FREE 137	7.19E+04	4.857	2.93E+04	7.30E+04	4.864	4.860	0.007
133	FREE 138	1.64E+04	4.215	5.68E+03	1.41E+04	4.150	4.183	-0.064
134	FREE 139	1.70E+03	3.230	7.89E+02	1.97E+03	3.293	3.262	0.063
135	FREE 140	< 40	-	-	-	-	-	-
136	FREE 141	4.33E+03	3.636	1.58E+03	3.94E+03	3.595	3.616	-0.041
137	FREE 142	1.07E+05	5.029	3.21E+04	8.00E+04	4.903	4.966	-0.126
138	FREE 143	1.77E+05	5.248	1.59E+05	3.95E+05	5.597	5.422	0.349
139	FREE 144	< 40	-	-	-	-	-	-
140	FREE 145	2.37E+04	4.375	3.88E+04	9.66E+04	4.985	4.680	0.610
141	FREE 146	1.42E+03	3.152	1.12E+03	2.79E+03	3.446	3.299	0.293
142	FREE 147	2.02E+03	3.305	1.34E+03	3.33E+03	3.522	3.414	0.216
143	FREE 148	9.62E+04	4.983	4.93E+04	1.23E+05	5.089	5.036	0.106
144	FREE 149	1.33E+05	5.124	1.24E+04	3.08E+04	4.489	4.806	-0.635
145	FREE 150	< 40	-	-	-	-	-	-
146	FREE 151	< 40	-	-	-	-	-	-
147	FREE 152	3.55E+03	3.550	1.21E+03	3.02E+03	3.480	3.515	-0.071
148	FREE 153	4.38E+04	4.641	2.59E+04	6.46E+04	4.810	4.726	0.169
149	FREE 154	< 40	-	-	-	-	-	-
150	FREE 155	2.14E+03	3.330	1.54E+03	3.84E+03	3.584	3.457	0.253
151	FREE 156	3.11E+04	4.493	6.59E+03	1.64E+04	4.215	4.354	-0.278