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EDDY OKOTH ODARI

aus (Geburtsort)
born in (place of birth)

KISUMU, KENYA

am (Tag an dem die Dissertation abgeschlossen wurde)
submitted on (day of finalization of the thesis)

1st July, 2014

Supervisors LMU:	Title, first name, last name
Habilitated Supervisor	Prof. Dr. Dr. h.c. Ulrich Koszinowski
Direct Supervisor	Dr. Hans Nitschko
3rd LMU Supervisor	Prof. Dr. Josef Eberle

Supervisor External:

Local Supervisor: Prof. Dr. Dr. Lutz Gürtler

Reviewing Experts:

1st Reviewer: Prof. Dr. Lutz Gürtler

2nd Reviewer: Dr. Hans Nitschko

Dean: Prof. Dr. med. Dr. h. c. M. Reiser, FACR, FRCR

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ESTABLISHMENT AND EVALUATION OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY (LAMP) FOR THE SEMI-QUANTITATIVE DETECTION OF HIV-1 GROUP M VIRUS IN BLOOD AND PLASMA

Affidavit

ODARI EDDY OKOTH

Surname, first name

Moosacher Strasse 81/611

Street

80809 München

Zip code, town

Germany

Country

I hereby declare, that the submitted thesis entitled

ESTABLISHMENT AND EVALUATION OF A LOOP-MEDIATED ISOTHERMAL

Thesis Title

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Abstract

The past decade has witnessed a dramatic increase of anti-retroviral treatment of Human Immunodeficiency Virus (HIV) infected patients in many African countries. Due to costs and sophistication of currently available commercial viral load assays, little attention has been paid to therapy monitoring through measurement of plasma viral load, a challenge that could reverse achievements already made against HIV/AIDS infection. Loop-mediated isothermal amplification (LAMP) has been shown to be simple, rapid and cost-effective, characteristics which make this assay ideal for viral load monitoring in resource limited settings.

The aim of this study was to establish and evaluate LAMP for quantitative detection of HIV-1 group M virus in blood and plasma.

Cell culture supernatants of HIV-1 subtype B (IIIB and MVP899-87) viruses were used to optimize reaction conditions and to test primer suitability. Together with HIV-1 M non-B subtypes, HIV-1 group O and HIV-2, the cell culture supernatants were used to evaluate the performance of LAMP, to generate a model for viral load estimation and to establish the limits of the assay. A panel of 467 clinical samples was analyzed (282 plasmas and 121 dry blood spots from Kenya and 112 plasmas from Germany) and the results obtained by LAMP were compared to those generated by the Abbott Real Time HIV-1 assay, an established commercial viral load quantification test. A linear regression equation was generated from time to detection values and used to estimate the viral loads of the samples by the LAMP assay. Kenyan samples were tested in Nairobi and Munich.

LAMP primers targeting the integrase of the *pol* gene were found to be the most suitable compared to further 3 primer sets tested. Lower limit of detection (LLOD) of 1,200 copies/mL and lower limit of quantification (LLOQ) of 9,800 copies/mL were determined as suitable thresholds for quantitative estimations of the LAMP viral loads. Sensitivities of 82 and 86% (Kenyan samples) and 93% (German samples) and specificities of 99 and 100% were realized with plasma samples. The study also realized a sensitivity of 76% and specificity of 77% with dry blood spot samples from Kenya.

In conclusion, LAMP assay shows obvious potential for diagnostic application in semi-quantification of HIV-1 group M viral load in resource limited countries. However there is a need for further improvement of primers in respect to detection of HIV-1 non-B viruses and evaluation of dry blood spot samples to ensure that more reliable results are obtained.

Key words: LAMP assay, HIV integrase primer, semi-quantitative, viral load, Kenya.

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Abbreviations and acronyms

AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-retroviral therapy
BIP	Backward inner primer
Bp	Base pair
***Bst* polymerase**	Polymerase enzyme from *Bacillus stearothermophilus*
CCR5	C-C chemokine receptor type 5
CD4	HIV-1 cluster of differentiation 4 glycoprotein
CI	Confidence interval
CRF	HIV-1 Circulating recombinant form
CV	Coefficient of variation
DBS	Dry blood spot
DD	Detection dye
dH_2O	distilled water
DNA	Deoxyribonucleic acid
DPS	Dry plasma spot
EC 50	Effective concentration 50
EDTA	Ethylene diamine tetra acetic acid
Env	HIV-1 envelope gene
FDA	Food and drug administration
FIP	Forward inner primer
FTA	Fast technology for analysis of nucleic acids
Gag	HIV-1 group-specific antigen gene
Gp41	HIV-1 glycoprotein with molecular weight 41,000
HAART	Highly Active Anti-retroviral Therapy
HiGp	Primer set targeting HIV gp41 of the *env* gene
HIV	Human Immunodeficiency Virus
HiInteg	Primer set targeting HIV integrase of the *pol* gene
HiLtr	Primer set targeting HIV LTR region
HiP	Primer set targeting HIV p24 of the *gag* gene
HNB	Hydroxynaphthol blue
HXB2	HIV-1 virus strain HXB clone 2
IN	integrase
K65R	Mutation of lysine to arginine in position 65
KEMRI	Kenya Medical Research Institute
LAMP	Loop-mediated isothermal amplification
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LTR	Long terminal repeat
MVPI	Max von Pettenkofer-Institute
ND	Not detected
NIAD/NIH	National institute of allergy and infectious diseases/ National

	institutes of health
NNRTI	Non-nucleoside reverse-transcriptase inhibitors
NPV	Negative predictive value
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitors
NYK	Sample obtained from Nyumbani diagnostic laboratory in Kenya
p24	HIV-1 core protein with molecular weight 24,000
PCR	Polymerase chain reaction
PMTCT	Prevention of mother-to-child transmission
pol	HIV-1 polymerase gene
PPV	Positive predictive value
qPCR	Quantitative PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPMI 1640	Roswell park memorial institute. *A formulation that supports growth of cultured cells including fresh human lymphocytes*
RT	Reverse transcriptase
rt-LAMP	Reverse transcription LAMP
rt-PCR	Reverse transcription PCR
SD	Standard deviation
SLS	Sodium lauryl sulfate
SIV	Simian Immunodeficiency Virus
***Taq* polymerase**	Polymerase enzyme from *Thermus aquaticus*
Tt	Time to threshold detection
U3	HIV-1 3'unique region
UNAIDS	The joint United Nations programme on HIV/AIDS
Vif	Virus infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X (HIV-1, SIV)
WHO	World Health Organization
WHO/TDR	World Health Organization/Tropical Diseases Research

Chapter 1

1 Introduction, justification and objectives of the study

1.1 Introduction

The identification of the first patients with Acquired Immunodeficiency Syndrome (AIDS) in 1981, and the subsequent proof of Human Immunodeficiency Virus (HIV) as the causative agent of AIDS by the Medical Scientific Community in 1984, marked the beginning of the fight against the spread of HIV/AIDS. The identification of HIV and its roots of infection thus became the first step in the process of eliminating transmission of the virus through sexual contacts, transfusion of blood and blood products, creating rational policies for control and prevention of infection by the different means of transmission, and designing efficient anti-retroviral therapies (Gallo and Montagnier, 2003).

Since the introduction of Highly Active Anti-Retroviral Therapy (HAART) for treatment of HIV/AIDS infection in 1995, millions of lives have been saved globally. Patients receiving this combination therapy continue to report decreased viral loads and increased CD4 T-lymphocyte counts. Thus the life expectancy among patients who start treatment at an early stage has moved closer to the general mortality rate of HIV negative persons (Johnson et al., 2013).

Despite the challenges at the beginning, a gradual increase in political commitment has been shown within the past 10 years by many governments in Sub-Saharan Africa, the continent with the highest burden of HIV/AIDS infection. Coupled with an increase in international funding within the same period, the increased level of political commitment has led to the rise in access to HIV/AIDS treatment in this region. Currently the number of people on anti-retroviral therapy (ART) stands at 7.1 million up from 1 million which was recorded in 2005 (UNAIDS, 2013). More than half of this number includes those patients who are in immediate need of treatment. However, despite this success, little attention has been paid to the possibility of the emergence and spread of drug resistant HIV strains and their implications to the public health systems.

As opposed to the developed world where management of combination therapy depends on: specialist care for individual patients (which includes drug resistance testing to guide on the choice of drug regimen), frequent monitoring of plasma viral load for prompt detection of treatment success or failure and a wide range of treatment regimens and options to choose from (Department for Health and Human Services, 2011), the developing world still has major limitations. Resource limited countries depend mainly on the public health approaches which include a decentralized service delivery, empirical first and second line anti-retroviral combination therapy and clinical or

immunological definitions of treatment failure in settings where plasma viral loads cannot be ascertained (Hamers et al., 2012a; World Health Organization, 2010a).

Reliance on immunological capacity through CD4 cell count, although successful, is not sufficient since CD4 cell count is a slowly changing parameter that gives insight into the function of the immune system. It is an assay which measures residual immune fitness and not viral replication in response to ART. Viral load measurement on the other hand, is a sensitive parameter mainly influenced by clearance of HIV from plasma by the immune system, efficiency of ART and drug combinations, compliance of the patient to drug regimen, and other conditions such as other infections or malnutrition which affect the competence of the immune response to control HIV.

With a constant reduction in international funding, African national health systems are likely to be faced with a lot of limitations which could lead to development of drug resistance. The current limitations include widespread use of low cost drugs which in some cases are of sub-optimal quality e.g. sometimes the use of nevirapine monotherapy in HIV prevention of mother-to-child transmission (PMTCT) and stavudine as part of first line treatment (Wainberg et al., 2011). Other limitations include limited access to monitoring of plasma viral load (Hosseinipour et al., 2009; Sigaloff et al., 2011), treatment interruptions when drug supplies run out (Oyugi et al., 2007; Pasquet et al., 2010), sub-optimal or non-adherence to treatment (Sethi et al., 2003) and frequent drug to drug interactions such as nevirapine and rifampicin in HIV patients co-infected with tuberculosis (Boulle et al., 2008) undergoing treatment.

Benefits of routine blood plasma viral load monitoring, which include avoiding unnecessary ART switches hence avoiding accumulation of drug resistant strains are increasingly being accepted by many stakeholders. The high costs of implementation and sustainability however remain a major point of concern, since any resources allocated to laboratory monitoring could divert funds away from expanding access to treatment thereby impinging on healthcare delivery. It is therefore important to develop and establish assays for viral load monitoring that are simple and cost effective for use in resource limited countries. This study investigates the performance of a loop-mediated isothermal amplification (LAMP) assay as a simple and cost effective assay that could potentially be used for quantitative determination of HIV plasma viral load in patients within resource limited settings.

1.2 Justification of the study

Studies estimate that between 10-24% (Barth et al., 2010; Hamers et al., 2012c) of HIV infected patients have detectable plasma viral load during first line therapy. However, switching rates to the

next line therapy is still low (Onyedum et al., 2013; World Health Organization, 2011) in resource limited settings, partly due to poor sensitivities of clinical and immunological criteria for detection of treatment failure (Sigaloff et al., 2011). It is also estimated that poor specificity of these criteria could lead to more than 50% of the switches being unnecessary (Sigaloff et. al., 2011). Unnecessary switching (from first line treatment) will rapidly exhaust the drug combination options available and unnecessarily increase the cost of treatment, since the cost of second line treatment is more than double that of the first line treatment (Clinton HIV AIDS Initiative, 2009). Increased cost would therefore mean that for every treatment of a wrongful switch to the second line treatment, one to two patients would go without treatment due to lack of drugs as a result of a limited budget (Clinton HIV AIDS Initiative, 2009). Therefore proper monitoring of plasma viral load could potentially save developing countries up to 15 – 30% of the costs of long term HIV treatment by mitigating high costs associated with unnecessary switching to second line regimen (Hamers et al., 2012b).

Apart from avoiding unnecessary switches, benefits of plasma viral load monitoring include supporting adherence surveillance (since lapses in treatment can quickly be identified) and identifying patients with accumulating drug resistant strains (Cohen et al., 2011; Wilson et al., 2009). Although these benefits are accepted, debate on high costs of implementation and sustenance by the resource limited countries still persists and in many cases favor clinical and immunological parameters, mainly CD4 cell count. CD4 cell count, as already discussed in page 2, only measures immune fitness and not viral replication in response to ART. Furthermore, studies carried out in the developed world have shown that measurement of CD4 cells have limited benefits in patients with suppressed viral loads (Ledergerber et al., 2004; Phillips et al., 2002).

Scientific advancements so far, such as use of dried blood spots (DBS) for sample storage and transportation, development of relatively simple assays for HIV detection continue to provide an opportunity for improvement and development of assays which would further reduce the costs associated with plasma viral load monitoring.

Therefore the threat of an emergence of drug resistant strains and high cost of monitoring treatment which could potentially reverse the gains made in the fight against HIV/AIDS, justifies the need for a simple, cost effective and readily available assay for use in monitoring plasma viral load in resource limited settings.

This study thus evaluated whether LAMP assay, regarded as simple and cost effective, would have the potential for use in quantitative detection of HIV-1 viral load in patients undergoing treatment within resource limited settings.

1.3 Objectives of the study

1.3.1 Broad objective

To establish and evaluate a LAMP assay for quantitative detection of HIV-1 group M virus RNA in blood and plasma.

1.3.2 Specific objectives

1. To compare the performance of primers designed to amplify 4 regions of the HIV genome, namely LTR, p24 in the *gag* gene, integrase in the *pol* gene and N-terminus of gp41 in the *env* gene.
2. To refine primers and cycling conditions during LAMP adaptation process.
3. To analyse performance characteristics of LAMP using cell culture supernatants of HIV-1 viruses from patients of different African regions and whole blood, plasma samples and dry blood spots (DBS) from Munich and Nairobi.
4. To evaluate the LAMP assay under field conditions in Kenya for HIV detection and viral load measurements

Chapter 2

2 Literature review

2.1 Human Immunodeficiency Virus (HIV)

2.1.1 Historical perspective

Human Immunodeficiency Virus, the virus that causes AIDS, was first discovered in 1983 (Barre-Sinoussi et al., 1983; Broder and Gallo, 1984; Gallo et al., 1983) and identified as lymphadenopathy-associated virus (LAV) by Montagnier's group and as human T-lymphotropic virus (HTLV-III) by Gallo's group. Montagnier's group in their virus characterization showed that this new virus had core proteins which were immunologically different from those of HTLV-1 (Basavapathruni and Anderson, 2007). Another research group in San Francisco in 1984 reported the isolation of a similar virus within symptomatic and asymptomatic patients from AIDS risk groups. They named this virus AIDS-associated retrovirus (ARV) (Levy et al., 1984). In 1986, the International Committee of Viral Taxonomy (ICVT) renamed all the three different viral isolates Human Immunodeficiency Virus (HIV-1) (Coffin et al., 1986), after it was demonstrated by nucleic acid sequence analysis of HTLV-III, LAV-1 and ARV that all three belonged to the same virus family (Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985).

2.1.2 Virus classification and genome morphology

2.1.2.1 Classification

Human Immunodeficiency Virus belongs to the family *Retroviridae* (Fauquet and Fargette, 2005) and as such is a RNA virus which codes for the enzyme reverse transcriptase. This enzyme transcribes the viral genomic RNA into DNA which then integrates into the genome of the host cell (Fauci, 1988) during the HIV life cycle.

Within the *Retroviridae* family, the virus is a member of the genus lentivirus (Fauquet and Fargette, 2005). Lentiviruses have a long incubation period between infection and the occurrence of disease (Levy, 1993). Lentiviruses are also known to cause immunodeficiency in their hosts in addition to slow progressive wasting disorders, neurodegeneration and death (Haase, 1986; Haase et al., 1990). These viruses include feline immunodeficiency virus infecting cats, visna virus infecting sheep, caprine arthritis-encephalitis virus infecting goats and simian immunodeficiency viruses infecting non-human primates (Gonda et al., 1985; Haase, 1986; Stowring et al., 1979; Temin, 1988; Temin, 1989).

2.1.2.2 Genome structure

Lentiviruses are distinguished from other members of the *Retroviridae* family by the complexity of their genome. HIV, like other lentiviruses contains three main structural genes, *gag, pol* and *env* (Figure 1). These genes contain information needed to code for the necessary structural proteins and enzymes (Los Alamos National Laboratory, 2008a). Apart from the three essential structural genes, HIV further contains complex regulatory genes *tat, rev, nef*, whose function is to regulate synthesis of proteins which control the ability of HIV to infect cells (infection), produce progeny viruses (replication), or cause disease (pathogenicity) (Los Alamos National Laboratory, 2008a). Other auxiliary genes include *vif, vpr* and *vpu* (or *vpx* in case of HIV-2) (Greene, 1991; Los Alamos National Laboratory, 2008a), and have also been referred to as accessory proteins. Although the latter have not been absolutely necessary for virus propagation in tissue culture, these accessory proteins have been shown to be conserved in the different isolates suggesting that their role *in vivo* is important. Their functional importance has been shown in pathogenicity (Kluge et al., 2013; Sharp and Hahn, 2011). The Long Terminal Repeats (LTRs) are found at the 5' and 3' ends and serve as a DNA sequence flanking the genome of integrated proviruses. The LTR contains important regulatory regions, especially those for transcription initiation and polyadenylation (Krebs et al., 2001).

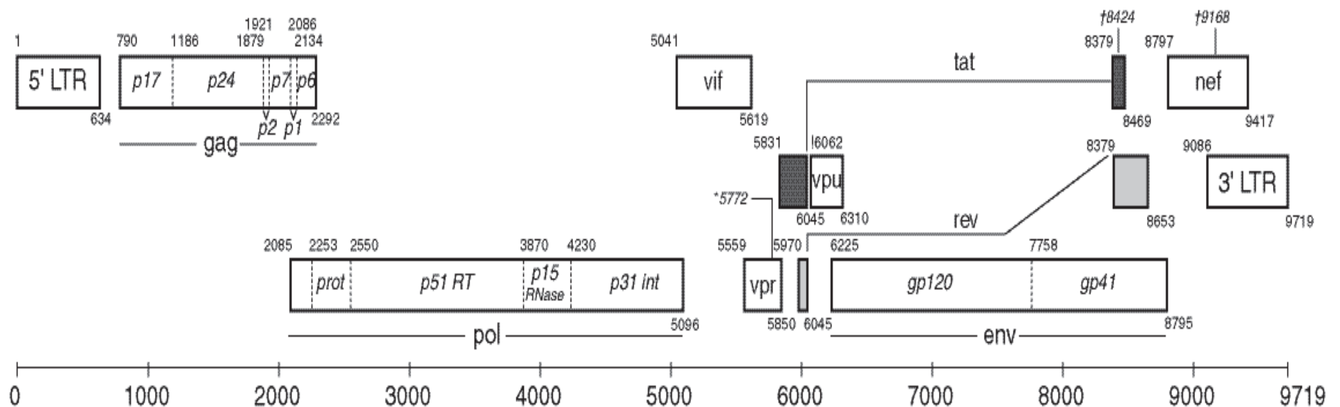


Figure 1. HIV genome structure. Structural genes *gag, pol* and *env*, regulatory genes *tat, rev, nef* , auxiliary genes *vif, vpr, vpu* and their positions within the HIV-1 genome of HXB2 strain (the whole genome consists of 9719 bp). Adapted from http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html

2.1.3 Epidemiology

2.1.3.1 Distribution of HIV types

Based on genetic variability (Robertson et al., 1995), two types of human immunodeficiency viruses (HIV-1 and HIV-2) have been identified. Both can be transmitted by seminal or vaginal secretions through sexual intercourse (Ho et al., 1984; Zagury et al., 1984), through transfusion of blood or blood products (Jones et al., 1992; Selik et al., 1993; Shrestha, 1996) sharing needles as seen in

intravenous drug use or through tattooing and scarification. Perinatal or vertical transmission from HIV-positive mother to the child (World Health Organization, 2010c) is another route and occurs during pregnancy, vaginal childbirth or through breastfeeding. HIV-2 is less easily transmitted and is characterized by a longer period between infection and progression to AIDS (Kanki et al., 1999; Marlink et al., 1994; Whittle et al., 1994) compared to HIV-1.

Although HIV-1 M with its subtypes has a global distribution, HIV-2 is mainly restricted to west Africa and India with some cases reported in Angola, Mozambique and Brazil (Marx et al., 2001; Santiago et al., 2005).

2.1.3.2 Global distribution of HIV-1 groups and subtypes

Four groups have been identified and are currently used to classify HIV-1 virus: group M "Major", O "outlier", N "Non M, non O" (Charneau et al., 1994; De Leys et al., 1990; Gurtler et al., 1994; Simon et al., 1998) and group P (Plantier et al., 2009; Vallari et al., 2011).

Groups O, N and P virus strains are more restricted to west and central African equatorial regions (the Congo basin), while group M is distributed world wide. Different studies have however shown sporadic cases of group O and N outside west and central Africa (Delaugerre et al., 2011; Gurtler et al., 1996; Rayfield et al., 1996; Sullivan et al., 2000) possibly caused by viral migration in man, since the majority of the cases had contact with west-central Africans (Delaugerre et al., 2011; Quinones-Mateu et al., 2000).

Group M therefore accounts for the large majority of HIV infections worldwide. This group is further divided into nine genetically distinct subtypes: A, B, C, D, F, G, H, J and K (Janssens et al., 1994a; Janssens et al., 1994b; Janssens et al., 1994c; Janssens et al., 1994d; Kostrikis et al., 1995; Leitner et al., 1995; Louwagie et al., 1995; Louwagie et al., 1993; Myers et al., 1992). When two viruses of different subtypes interchange their genetic material in the process of recombination, a new hybrid virus may result (Burke, 1997). When a hybrid (recombinant) virus infects more than one person, it is defined as a circulating recombinant form (CRF) (Burke, 1997). These CRFs have frequently been documented in HIV infected populations (Carr et al., 1996; Gao et al., 1996; Li et al., 2013; Murphy et al., 1993; Wei et al., 2014). Subtypes initially named E and I were subsequently grouped as CRFs (Los Alamos National Laboratory, 2008b).

Global estimates of HIV-1 group M virus subtypes show that subtype C accounts for approximately 50% of all infections worldwide followed by subtypes A, B, D and G accounting for 12%, 10%, 3% and 6%, respectively. Subtypes F, H, J and K combined together account for only 1% of infections

among the 9 major subtypes identified. Some studies have reported that pure subtypes account for 82% whereas the CRFs account for the remaining 18% (Hemelaar et al., 2006) of HIV-1 infections. Circulating recombinant forms CRF01_AE and CRF02_AG each are estimated to account for 5% of infections while CRF03_AB accounts for 0.1%. All other remaining recombinants account for the final 8% of infections (Hemelaar et al., 2006). A total of 65 major CRFs have been published to date (Los Alamos National Laboratory, 2013; Wei et al., 2014) and one inter-circulating recombinant form (Inter-CRF) designated CRF61_BC (composed of CRF07_BC and CRF08_BC) has already been identified in China (Li et al., 2013).

2.1.3.3 HIV-1 group M subtypes and disease progression

In a study done among female sex workers in Senegal infected with subtypes A, C, D and G (Kanki et al., 1999), it was realized that women infected with a non-A subtype were eight times more likely to develop AIDS than those infected with subtype A. This study therefore suggested a possible difference in pathogenicity and the rate of progression to AIDS for the different HIV-1 group M subtypes.

Other studies have shown that infection by specific subtypes influences the rate of disease progression and eventual death. In a study in Uganda, it was realized that persons infected with HIV-1 subtype D or recombinant strains incorporating subtype D developed AIDS sooner than those infected with subtype A, and in the absence of ART these patients also died earlier (Kiwanuka et al., 2008). This Ugandan study associated the virulence of subtype D to its more effective binding to the immune cells. A similar study among Kenyan women also showed that those infected with subtype D had more than twice the risk of death over six years compared to those infected with subtype A (Baeten et al., 2007), despite similarity in their plasma viral loads. A study done to evaluate the survival patterns of adults in Thailand 8-14 years after HIV-1 subtype E (CRF01_AE) infection (Nelson et al., 2007) showed a similar survival pattern of these individuals comparable to those individuals infected with HIV-1 group M subtype D in Africa. In their findings, Nelson and colleagues also reported that CRF01_AE had a higher rate of disease progression compared to subtype B. The latter finding could probably point towards the effect of increasing pathogenicity and recombination of an infecting virus on the time to disease and AIDS-related death, since B and D are grouped as sub-subtypes with very closely related genetic patterns (Robertson et al., 1999). This faster progression to AIDS and AIDS-related death has also been reported in patients from Guinea-Bissau infected with a circulating recombinant form A3/A2 (Palm et al., 2014). The latter study has so far reported the fastest rate of progression from HIV infection to disease and AIDS-related death.

2.1.3.4 HIV-1 group M subtypes and transmission of infection

Until now the association between HIV subtype and the transmission route of infection has not conclusively been evaluated. However, some studies (van Harmelen et al., 1997) have reported that subtype B is mainly transmitted among homosexual men compared to subtype C which is mainly found in the heterosexual setting. This assertion could be strengthened by the global subtype distribution where subtype B is predominantly common in the western world within the gay communities. Among the Thai population, it has also been reported that CRF01_AE is transmitted more easily in heterosexual relationships compared to subtype B. Whether any biological factors influence these observations has continuously been a subject of debate (Bhoopat et al., 2001; Dittmar et al., 1997; Essex, 1996; Pope et al., 1997).

It is also not clear as yet whether infection by a specific HIV subtype influences the rate of vertical transmission from mother to child). A matched case-control study addressing whether viruses with different long terminal repeat (LTR) subtypes were transmitted equally vertically among a Tanzanian population (Blackard et al., 2001) reported a higher rate of mother to child transmission of subtype C compared to subtypes A and D. In this study, the rate of subtype A transmission was 3.2 times higher than that of subtype D. However a study done in western Kenya among 414 women (Yang et al., 2003) identified higher rates of mother to child transmission among women infected with subtype D compared to those infected with subtype A. Another study carried out in a Tanzanian population targeting the *env* gene (Renjifo et al., 2004) also identified a higher rate of transmission of subtype C compared to a combined rate of subtype A and D. Other studies have however shown no significant association between HIV subtype and the rate of mother to child transmission (Martinez et al., 2006; Murray et al., 2000; Tapia et al., 2003).

2.1.3.5 HIV-1 group M subtypes and response to anti-retroviral therapy

Although different studies have attempted to address this issue, several challenges have been fronted with the results obtained from various studies. In their study on response to therapy among 113 children infected with seven different subtypes (A (15%), B (41%), C (16%), D (9%), F (5%), G (2%), H (1%)), CRF01_AE (5%) and CRF02_AG (6%), the Pediatric European Network for Treatment of AIDS (PENTA 5 trial) observed no significant difference after 48 weeks of treatment (Pillay et al., 2002). This PENTA 5 study however presented with a challenge of low statistical power and thus a difficulty in ruling out minor differences between subtypes. It is also worth noting that only four different sets of drugs, zidovudine, lamivudine, abacavir, and nelfinavir were used. In another study evaluating the virological response to treatment in Europeans infected with subtype B and Africans infected with non-B subtype (Frater et al., 2002), there was no difference in time to

undetectable viral load as well as recovery of the CD4 cells. However, a significant difference was observed with viral load over time where the African group exhibited a continuous increase in viral load over the 9 months period, which suggested a higher therapy failure. Although the authors concluded the difference to be mainly due to poor adherence among the African group, a number of limitations could be cited from this study. Subtype was mainly presumed from the ethnic and epidemiological data, after a confirmation of only 60% of the 97 Africans and 30% of the 265 Europeans. Hence in depth conclusion of subtype responsibility in the treatment failure could not substantially be ascertained. Furthermore, there was an imbalance in the two groups, including the types of regimen used, transmission risk groups, CD4 cell counts and gender (Pillay et al., 2002).

2.1.3.6 HIV-1 group M subtypes and resistance to anti-retroviral therapy

The majority of drug resistance testing has been performed on subtype B virus, however it is estimated that more than 90% of resistances occur in the RT within the non-B subtypes (Lessells et al., 2012). K65R (mutation of lysine to arginine in position 65) induced resistance to TDF and NRTI is an example of a clinically relevant mutation that emerges more frequently and more rapidly in subtype C virus compared to subtype B (Lessells et al., 2012). Further, possibilities of genetic differences among subtypes exist which may yield differential patterns in the mutations that aggravate resistance in response to ART pressure. HIV-1 subtype genetic variations have been estimated at 35% and in some regions of the genome, e.g. the *env* gene, these variations have been reported as high as 40%, whereas in the regions such as the *gag* and the *pol* (IN) genes these variations have been reported as low as 8 to 10% (Brenner, 2007). Since changes in codon sequences at positions associated with drug resistance mutations could predispose viral isolates from different subtypes to encode different amino acid substitutions, it is thus possible that genetic diversity in HIV-1 could influence the type of resistance mutations (Brenner, 2007; Kantor, 2006). Brenner and colleagues also assert that this diversity may affect the degree of cross resistance to ARTs of the same drug class, with a potential impact on clinical outcome, preservation of immunological responsiveness and virologic failure (Brenner, 2007).

A clinical trial among pregnant women taking a single dose nevirapine for PMTCT showed an overall resistance of 69% to nevirapine with subtype C virus while subtypes D, A and CRF02_AG showed 36%, 19% and 21% resistance mutations respectively in various studies (Chaix et al., 2006; Eshleman et al., 2005; Toni et al., 2005). Several studies have also shown variations in levels of resistance for the different known subtypes. The recombinant form CRF02_AG has been shown to be more susceptible to the action of nelfinavir and ritonavir than subtypes C and F whereas subtype G was

shown to be more sensitive to tipranavir and lopinavir than other subtypes (Marcelin et al., 2008). Subtype C on the other hand was shown in different studies to have an increased risk of developing resistance to tenofovir (at K65R) than other subtypes (Brenner et al., 2006; Doualla-Bell et al., 2006; Miller et al., 2007).

Mutational variations in the HIV genome are the major factors considered in assigning different subtypes and identifying resistant or emerging strains. These variations therefore continue to play a major role in the HIV pandemic, and knowledge of HIV subtype distribution coupled with the continuous global molecular epidemiology surveillance on emerging strains or imported strains remain fundamental..

2.1.3.7 HIV infection in Sub-Saharan Africa

Although still a home to 70% of all HIV new infections globally, Sub-Saharan Africa has seen a steady decline, with annual numbers of new infections among adults declining by up to 34% since 2001 (UNAIDS, 2013). The high level of political commitment currently seen in this African region and the substantial amount of international funding have both led to a drastic scale up in access to HIV treatment (World Health Organization, 2011). But even with this success, there is still a major challenge of a potential spread of drug resistant mutants since little attention has been paid to the emergence of drug mutant strains and their implication to public health.

2.1.4 HIV treatment

2.1.4.1 Classes of HIV drugs

Currently there is still no drug available for complete cure of HIV infection (thus eliminating the virus from all cells of the body) (Passaes and Saez-Cirion, 2014), a fact that could remain for the next decade. The aim of treatment is therefore to reduce the amount of viral particles to undetectable levels in order to help the body restore the weakened immune system. The final result is that an individual leads a normal life even with low amounts of virus in circulation. Currently these drugs are categorized into six different classes with various drugs in each category already approved by the U.S Food and Drug Administration (Food and Drug Administration (FDA), 2013). These classes include (1) Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) (2) Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) (3) Protease Inhibitors (PIs) (4) Fusion Inhibitors (5) Entry Inhibitors (CCR5 co-receptor antagonist) and (6) HIV Integrase Inhibitors. Each of these classes of drugs is designed to target a specific stage in the HIV replication cycle (NIAD/NIH, 2012).

Nucleos(t)ide Reverse Transcriptase Inhibitors (NRTIs) were the first class of drugs to be approved by the FDA (Young, 1988). These drugs inhibit the synthesis of viral DNA by the action of reverse transcriptase, the viral enzyme that copies viral RNA into DNA. They are mainly nucleoside analogues which bear structural resemblance to the natural nucleoside components of DNA (adenosine (A) and guanosine (G), thymidine (T) and cytosine (C)). These dideoxy-analogues are triphosphorylated within the cell. During the replication process, reverse transcriptase enzyme fails to distinguish between the phosphorylated nucleosides and their natural counterparts hence using the drugs, instead of the nucleosides, in the synthesis of DNA. The drugs are therefore incorporated into the newly synthesized strand thereby preventing addition of further nucleotides (http://hivinsite.ucsf.edu/InSite?page =ar-drugs-about). Presently used drugs include: lamivudine (3TC), zidovudine (AZT), emtricitabine (FTC), abacavir (ABC), dideoxyinosine (ddI), stavudine (d4T) and the nucleotide analogue tenofovir disoproxil fumarate (TDF).

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) also inhibit the synthesis of viral DNA. As opposed to NRTIs which act as nucleoside analogues (false nucleosides) NNRTIs bind to reverse transcriptase hence inhibiting the enzyme's activities. Unlike the NRTIs this class of drugs does not inhibit the RT of related retroviruses such as HIV-1 group O and P, HIV-2 and simian immunodeficiency virus (SIV) (Kohlstaedt and Steitz, 1992; Witvrouw et al., 1999). These NNRTI include: rilpivirine (RPV), etravirine (ETV), delavirdine (DLV), efavirenz (EFV) and nevirapine (NVP).

Protease inhibitors (PIs) bind to the active site of the protease enzyme hence preventing the processing of viral proteins into functional size and conformation. HIV-1 protease is the enzyme which cleaves *gag-pol* polyprotein, *gag* and *pol* precursors during maturation of the virion (Miller, 2001; Park and Morrow, 1993). Although the viral particles are still produced even when the protease enzyme is inhibited, these particles cannot infect new cells. Protease inhibitors include: tipranavir (TPV), indinavir (IDV), saquinavir mesylate (SQV), lopinavir (LPV), ritonavir (RTV), fosamprenavir (fAPV), darunavir (DRV), atazanavir (ATV) and nelfinavir (NFV).

Fusion Inhibitors prevent the virus from entering target cells. The drug binds to the viral gp41 envelope protein known for its involvement during entry of the virus into the cell. The drug thus blocks interactions between neighboring regions of the gp41 molecule. It eventually interferes with the conformational change (folding) of the envelope molecule required for fusion with the target cell membrane. Only one peptide-based drug known as enfuvirtide (T-20) currently represents this class.

Entry Inhibitors (CCR5 co-receptor antagonist) or chemokine co-receptor antagonists prevent viral entry into the target cells. They bind to co-receptors (either CCR5 or CCR2) on the surface of T-helper CD4 cells hence blocking entry. As opposed to other classes of drugs which target the virus, this class of drugs targets a human cell-surface protein. They include maraviroc (MVC) and cenicriviroc (CVC), the latter is currently under clinical evaluation.

Integrase inhibitors (INIs) mainly target the strand transfer reaction hence have also been known as integrase strand transfer inhibitors (InSTIs) (Espeseth et al., 2000; Hazuda et al., 2004; McColl and Chen, 2010). The drugs bind to the integrase enzyme thus interfering with incorporation of the already reverse-transcribed pro-viral DNA into the chromosomes of the host cell. INIs include raltegravir (RGV), recently approved dolutegravir (DTG) and elvitegravir (EGV) (Messiaen et al., 2013).

2.1.4.2 Highly Active Anti-retroviral Therapy (HAART)

HAART describes the parallel use of a combination of three or more anti-HIV drugs in the treatment of HIV infection. Use of a combination therapy reduces the risk of development of resistance to the drugs as opposed to single drug therapy, making treatment effective in the long run. Guidelines for HAART have been actualized (World Health Organization, 2013b) where stavudine (d4T) which had initially been used together with lamivudine (3TC) and nevirapine (NVP) mainly due to its low cost (Gilks et al., 2006), is currently replaced by tenofovir (TDF) in the first line therapy. First line therapy which traditionally consists of 1 non-nucleoside reverse transcriptase inhibitor (NNRTI) and 2 nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), thus consists, for example, of a fixed-dose combination of tenofovir (TDF), emtricitabine (FTC) and efavirenz (EFV) as a single pill (World Health Organization, 2013b). First line therapy is usually initiated at the beginning of treatment of HIV infection. However, due to different factors including the side effects and the development of resistance to first line therapy, a second line treatment module is recommended. Second line therapy mainly consists of a ritonavir-boosted protease inhibitor and two NRTIs. The choice of drugs used in each category will depend on different factors including, availability and the costs of the drug, number of pills to be taken daily, the side effects of the drugs and the type of laboratory monitoring.

Third line therapy is also known as the salvage or rescue therapy. This term describes treatment regimens for people who have very few or limited anti-HIV drug options due to resistance. They include patients recording failure in at least two previous anti-HIV regimens, or those patients proved to mount HIV drug resistance to at least one drug in each of the three major classes, mainly the

NRTIs, NNRTIs and PIs. True salvage or deep salvage is a term used to describe treatment of a person who has literally no treatment options. Most third line regimens consist of four or more drugs. It has been suggested that third line regimens should include new drugs likely to have anti-HIV activity, such as integrase inhibitors and second-generation NNRTIs and PIs (World Health Organization, 2010a). However it is important to note that in 2010 the World Health Organization made three major recommendations regarding management of multiple treatment failures, which included: national programs to develop policies for third-line therapy that consider funding, sustainability and the provision of equitable access to ART; third-line regimens to include new drugs likely to have anti-HIV activity, such as integrase inhibitors and second-generation NNRTIs and PIs and finally patients on a failing second-line regimen with no new ART options to continue with a regimen that is tolerated (World Health Organization, 2010a).

2.1.4.3 HIV treatment in Sub-Saharan Africa

As opposed to the developed economies where treatment and management of HIV infection is based on individualized specialist care, the resource limited countries mainly follow the World Health Organization (World Health Organization, 2010c) public health approach. Individualized specialist care in the developed world includes frequent monitoring of plasma viral loads for early detection of treatment failures, drug resistance testing which guide on the most appropriate regimen and a wider range of anti-retroviral drugs to choose from (Department for Health and Human Services, 2011). On the other hand, the WHO public health approach is based on a decentralized service delivery, empirical first and second line therapies, and empirical clinical or immunological definitions of treatment failure in the absence of information on plasma viral load (World Health Organization, 2010c).

Since roll-out of ART in Sub-Saharan Africa more than 15 years ago several countries have continued to use triple therapy in treatment of HIV infection. These countries however continue to meet various challenges which if not checked, have likelihood to aggravate the problem of drug resistance. As already mentioned, some of these challenges include continued use of low cost, substandard drug regimen. For example, until recently, stavudine has been used together with nevirapine as part of the first line regimen in HIV PMTCT (Wainberg et al., 2011). Other challenges have included treatment interruptions when stock or supply run out (Oyugi et al., 2007; Pasquet et al., 2010), restricted or no access to plasma viral load monitoring of treatment failures (Hosseinipour et al., 2009; Sigaloff et al., 2011), sub-optimal adherence to therapy due to various factors (Sethi et al., 2003) and frequent drug – drug interactions especially among patients co-infected with tuberculosis

undergoing treatment (Boulle et al., 2008). All these factors have a potential to necessitate the development of drug resistant strains in Sub-Saharan Africa.

2.1.4.4 HIV drug resistance

During replication, HIV, like any other retrovirus, uses the enzyme reverse transcriptase in the synthesis of viral DNA from the RNA genome (Freeman, 2007). Reverse transcriptase however, lacks proof reading capabilities and as such is unable to correct errors made during the replication process. This leads to mutations occurring within the viral genome (Freeman, 2007). High mutation rates have been recorded with HIV (Boyer and Hughes, 2000; Lewis et al., 1999; Mansky, 1996; Mansky and Temin, 1995; Rezende and Prasad, 2004; Roberts et al., 1988; Stuke et al., 1997; Svarovskaia et al., 2003) with a retroviral average of 1.5×10^{-5} mutations/bp/cycle (Mansky and Temin, 1994; Mansky and Temin, 1995; Parthasarathi et al., 1995; Pathak and Temin, 1990a; Pathak and Temin, 1990b). These mutations then result in circulating mutant strains, which in most cases would be destroyed by the immune system. Those that escape immune recognition continue to replicate and accumulate into quasi-species as a result of an increased probability of a virion developing an evolutionary selective advantage over other virions (Freeman, 2007). The quasi-species within HIV infected can easily adapt to their host (Domingo et al., 1997; Goodenow et al., 1989; Lemey et al., 2006; Vignuzzi et al., 2006; Wolinsky et al., 1996; Yamaguchi and Gojobori, 1997; Zhang et al., 1997) partly due to variations in the replicative fitness which enables a faster growth (Domingo et al., 1997; Tebit et al., 2007). Due to natural selection, virions with higher fitness are then selected while others are cleared by drug treatment and immune actions (Kozal, 2009). The virions that are able to escape the harmful effects of the drug then create an entirely new, drug resistant population. These virions replicate in the patient to a high load similar to that level of the viruses which circulatedin the patient before treatment. The patient thus experiences a decline in the effectiveness of treatment as the virus develops resistance leading to an increased amount of resistant variants. This process is known as acquired resistance.

The drug resistant variants selected during treatment failure thus have the potential to resist subsequent treatments with the same drugs, and therefore constitute a reservoir for onward transmission to newly infected persons (Hamers et al., 2012a) in a process known as transmitted resistance.

Without prompt identification, resistant strains will therefore be transmitted within a population, restricting treatment options and increasing the costs of treatment, as a large number of patients will have to be switched to the second line of treatment from the beginning. Scaling up of HIV treatment

in many African countries has also come with its equal share of increase in drug resistance (Aghokeng et al., 2011; Hamers et al., 2011; Price et al., 2011), mainly to NNRTIs (World Health Organization, 2010a; World Health Organization, 2010c) which constitute the first line treatment regimens. Hamers and colleagues in their study in six African countries (Hamers et al., 2011) estimated the rate of transmitted drug resistance to increase at 38% per year after the roll-out of ART, with pre-treatment failure found to double the failure to first line drugs and further acquisition of resistant variants within the first year of treatment. It is therefore important to monitor any emergence of HIV resistance within a population.

2.1.4.5 Viral load and CD4 Cells

Although WHO has established clinical and immunological parameters of monitoring treatment within the resource limited settings, an important challenge to many anti-retroviral programs is how to identify those patients with treatment failure and promptly switch them to second line therapy. As already mentioned, an estimated 10% to 24% of patients report detectable plasma viral loads during the first line treatment in Sub-Saharan Africa (Barth et al., 2010; Hamers et al., 2012c), however very low switching rates have been reported (World Health Organization, 2011), mainly due to poor sensitivity of these (clinical or immunological) parameters. Poor specificity of the parameters would also lead to unnecessary switches, putting a burden to the cost of treatment and exhaustion of available regimen. These two factors of poor sensitivity and specificity of the WHO established parameters would literally impinge on the successes achieved in Africa within the last decade.

One main challenge of HIV/AIDS treatment in Sub-Saharan Africa is adherence (Wilson et al., 2009) which is partly attributed to the high level of poverty and the life-style of some groups within the populations. Hence the concept of treatment monitoring becomes apparent as lapses potentially due to poor treatment adherence would quickly become apparent and patients at risk of developing resistance promptly identified (Cohen et al., 2011).

In the long run it is anticipated that treatment monitoring through plasma viral load and CD4 cell count in resource limited settings has a cumulative effect of saving 15-30% of the cost of treatment which would otherwise occur as a result of unnecessary switching to second line therapy (Hamers et al., 2012b).

2.1.4.5.1 CD4 cell count/measurement

High costs associated with current viral load assays have impinged on many African governments initiating viral load monitoring programs in the public health sector. With the support of international donor funding, e.g. Bill and Melinda Gates foundation, many African HIV programs currently rely on

CD4 cell counts. CD4 cell count, however, is an indirect parameter which mainly gives an insight into the residual function of the immune system and not viral replication in response to ART (see pages 2 and 3). The level of CD4 cells in the body is affected by other factors such as nutrition status and a variety of body conditions including the presence of other infections. CD4 cell count therefore has its limitations, such as failure to promptly detect patients failing treatment due to factors such as adherence or resistance. Besides, CD4 cell count has been reported to have limited benefits in patients with suppressed viral load (Ledergerber et al., 2004; Phillips et al., 2002).

2.1.4.5.2 Viral load monitoring

Several challenges still exist with the current nucleic acid based techniques in use. These challenges include among others the fact that these techniques still remain special techniques available only to particular facilities and laboratories because of the use of possibly toxic and unstable fluorescent dyes or due to the need to use special equipment for amplification and detection.

Polymerase Chain Reaction (PCR), which has a wide usage, has got intrinsic disadvantages of complex and multiple processes requiring a thermal cycler for the reaction and a time consuming post-PCR analysis, which potentially increases the risk of laboratory contamination as seen with nested PCR. The development of quantitative real-time PCR (qPCR) (Bustin et al., 2009; Gibson et al., 1996; Heid et al., 1996) has brought in several advantages over conventional PCR methods, including, rapidity, quantitative measurements, lower contamination rate, higher sensitivity, higher specificity and easy standardization (Mackay et al., 2002). Real-time qPCR however has disadvantages of complexity since the system requires an instrumentation platform consisting of a thermal cycler, computer, optics for fluorescence excitation and emission as well as data collection and analysis software, hence the need for high expertise and state of the art equipped laboratories. The machines are also very expensive and not within the purchasing powers of many laboratories in the developing or resource limited countries. Therefore even with the availability of commercial nucleic acid based tests, their use is limited to selected laboratory settings as they are expensive. For example, as at 2007, the kits ranged from U.S $ 50 to 100 per test (Dineva et al., 2007; Fiscus et al., 2006), which is comparable to a subsidized monthly HIV treatment cost of the same amount in Sub-Saharan Africa. These disadvantages related to cost, test performance time, computer capacity, extensive training and complex or dedicated laboratories for sample preparation and amplification, are an indication that the needs of test users are difficult to fulfill for viral load monitoring in resource limited settings. In these settings, cost and assay time pose considerable constraints for most testing and management strategies for HIV infected patients.

With these high costs, long time consumption and other disadvantages associated with the currently available nucleic acid techniques and viral load assays, it is important to develop more cost effective and easy to use assays for use in detecting HIV and monitoring viral load in patients undergoing treatment in resource limited settings. This is one way to ensure that the achievements made so far in the fight against HIV/AIDS in Sub-Saharan Africa will be maintained and improved.

2.2 Loop-mediated isothermal amplification (LAMP)

2.2.1 Test principle and LAMP primers

The LAMP assay was developed about 14 years ago (Notomi et al., 2000) and is currently evaluated and established for a variety of infectious agents. LAMP exhibits characteristics that are ideal for the development of a rapid, cost-effective nucleic acid-based test for detection of many infectious agents (Mori and Notomi, 2009). It is a one step isothermal amplification reaction that amplifies a target nucleic acid sequence utilizing a DNA polymerase with strand displacement activity, along with two inner primers, forward inner primer (FIP) and backward inner primer (BIP), and two outer primers forward 3 (F3) and Backward 3 (B3). These primers are specially designed for six specific regions within the target sequence. Two additional primers, loop forward (LF) and loop backward (LB) have been developed for enhanced specificity and reaction efficiency (Nagamine et al., 2002) and to accelerate LAMP reaction. A scheme of the primer targets in the DNA is shown in Figure 2a.

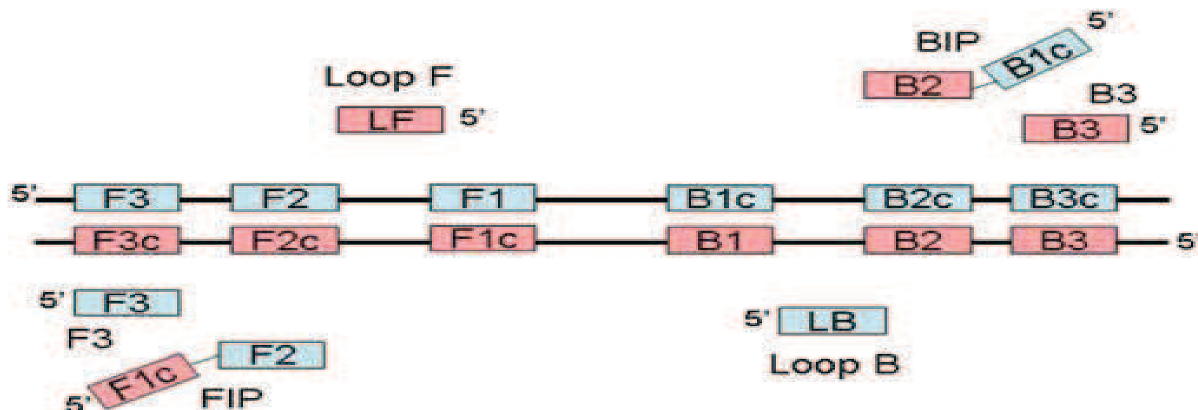


Figure 2a. LAMP primer targets. Schematic representation of loop-mediated isothermal amplification (LAMP) primers showing; inner primers FIP (F1c and F2) and BIP (B1c and B2) and the outer primers, designed at the region of F3 and B3. The loop primers Loop F and Loop B are designed to target between the regions F1c - F2c and B1c – B2c respectively (adapted from Mori and Notomi, 2009).

Isothermal reaction in LAMP starts with the displacement of the double strands of DNA by the polymerase enzyme and undergoes 8 important steps to form a "dumb-bell" structure which then undergoes a cyclic amplification process (Refer. http://loopamp.eiken.co.jp/e/lamp/principle.html).

The cyclic amplification is a complex (Figure 2b) process involving an exponential amplification of the original dumb-bell shaped stem-loop DNA by use of the inner primers (FIP and BIP). As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

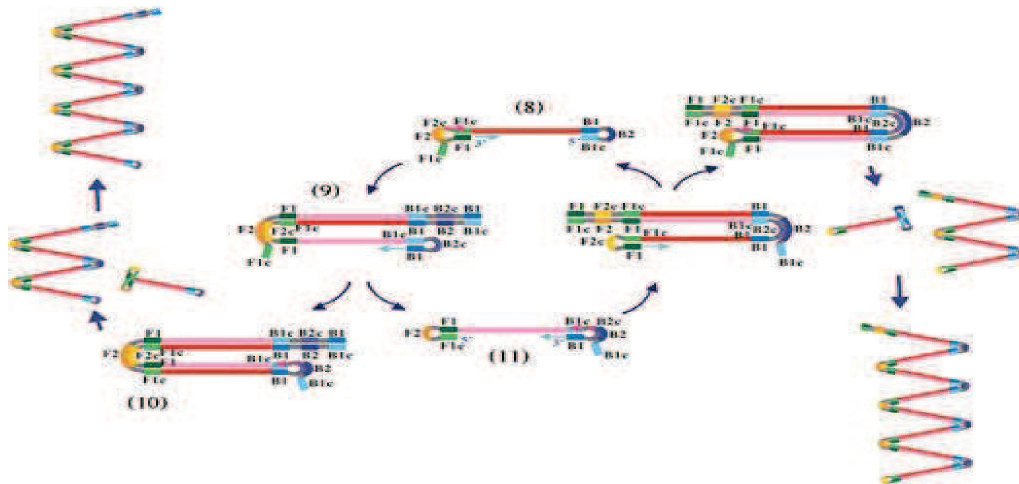


Figure 2b. Schematic representation of the cyclic step in the LAMP amplification process. Steps 9-11 start from the dumb-bell shaped stem loop generated in step 8, then undergo exponential amplification using inner primers (FIP and BIP); this leads to the formation of differently sized structures consisting of alternately inverted repeats of the target sequence on the same strand, resulting in cauliflower-like (shown at the upper left and lower right corners) structures. (*Copyright ©, 2005, Einken Chemical Co. ltd, Japan*).

2.2.2 Advantages of LAMP assay and its adaptation to detection of viruses

The LAMP technology has also been adapted for the detection of RNA viruses using reverse transcription loop-mediated isothermal amplification (rt-LAMP), simply through the addition of a heat stable reverse transcriptase enzyme (Hong et al., 2004; Kurosaki et al., 2007; Soliman and El-Matbouli, 2006; Yoshida et al., 2007), making it an ideal assay for HIV nucleic acid detection. A major asset of this procedure is that it is carried out under isothermal conditions. Operation and control of this assay does neither require sophisticated machinery nor highly equipped laboratory facilities, as the amplification reaction can be carried out in a heating block or a water bath, obviating the need for a thermal cycler. The whole procedure is also very simple and rapid. The amplification can be completed in less than one hour by incubating all the reagents in a single tube. Gene amplification products can be detected by agarose gel electrophoresis as well as by real-time monitoring in an inexpensive turbidimeter measuring the amount of magnesium pyrophosphate deposits. Gene copy number can also be quantified with the help of a standard curve generated from different concentrations of gene copy numbers plotted against time to positive signal with the help of a real-time turbidimeter (Parida et al., 2008).

This technology has been developed into commercially available detection kits for a variety of pathogens including bacteria and viruses (Mori and Notomi, 2009). In comparison to rt-PCR which is currently used for genome amplification of infectious agents in the developed world, this procedure has shown great similarities, giving a concordance of 85% on Japanese encephalitis virus detection (Parida et al., 2006). Different studies have successfully evaluated this assay for its adaptation in nucleic acid detection of HIV-1 group M viruses (Curtis et al., 2008; Hosaka et al., 2009). However, not until recently has rt-LAMP been shown to have potential for semi-quantitative analysis of Loa loa samples (Drame et al., 2014). Promising results have been published by rt-LAMP for quantification of HIV-1 strains circulating within a Chinese population (Zeng et al., 2014) and in HIV-2 viruses (Curtis et al., 2014).

Considering the advantages of rapid amplification, simple operation and easy detection, LAMP has potential applications for clinical diagnosis and management of HIV infection in developing countries without requiring sophisticated equipment or personnel with special training. Thus there is confidence that this assay, once established in a robust form, will work for detection of HIV-RNA and for the determination of viral load in HIV-1 patients in Africa, and further lend itself as a refined parameter for the proper monitoring of ART treated patients in the sub-urban regions of African countries.

Chapter 3

3 Materials and methods

3.1 Sample materials

3.1.1 Cell culture supernatants

Purified RNA from HIV-1 propagated in cell cultures at the Max von Pettenkofer-Institute was used during the optimization of the LAMP assay. Serial dilutions of IIIB and MVP899-87, representing HIV-1 group M subtype B, formed a panel that was initially used for the optimization process of the primers and the assay system. Other cell culture supernatants representing various HIV types, groups and subtypes included: 2 HIV-2 samples MVP11971-87 and MVP438-01; and 6 HIV-1 group O samples, MVP2000-01, MVP2901-94, MVP2549-95, MVP5267-95, FLI5066-03 and MVP 5180-91. HIV-1 group M non-B subtypes included: MVP8268A-98 (HIV M: A), V0715163B (HIV-1 M: A1), 3 samples MVP3777-97, MVP5739-98 and FLI810B2-07 (HIV M: C), MVP9619-94 (HIV-1 M:D), FLI4334A-99 (HIV M:CRF01_AE), V0634505 (HIV-1 M: F1), MVP7854F-94 (HIV-1 M:F2) and V1216136B (HIV-1 M:G). The HIV-1 groups N and P were not represented since these viruses are very rare and samples of the two groups were not available.

3.1.2 Clinical samples

Clinical samples were obtained either from HIV infected patients who were undergoing treatment at the time of the study or from HIV positive plasma samples archived in Nairobi and in Munich. Samples were categorized into whole blood samples, plasma samples and dry blood/plasma spot (DBS/DPS) samples. Part of the samples obtained from Kenyan patients in Nairobi was transported to the Max von Pettenkofer-Institute for further analysis.

Using a standard formula recommended by WHO/TDR (Banoo et al., 2010) a minimum total of 140 samples had been calculated to be sufficient for the study. However for a statistically suitable data set a total of 467 samples, including both HIV positive and negative were analyzed during the study. These samples included 234 HIV positive plasma samples from Kenya of which 87 were analyzed in Nairobi while 99 were analyzed in Munich. Others from Kenya included 121 HIV positive dry blood spot samples which were shipped to and analyzed in Munich and 48 HIV antibody negative samples obtained and analyzed in Nairobi. The HIV antibody negative samples were obtained from healthy patients attending or whose blood samples were sent to the Kenya Medical Research Institute (KEMRI) for routine medical checks for food borne infections. The final group of samples was a panel of 112 plasmas including HIV positive and negative samples obtained from patients undergoing

treatment and from persons undergoing routine testing in Munich. The permission to use these samples was obtained from the respective institutions.

3.2 Laboratory procedures

3.2.1 Optimization of LAMP assay using cell culture supernatants

3.2.1.1 Viral strains and reagents

Cell culture supernatant in-house positive controls (IIIB and MVP899-87) for HIV-1 group M subtype B, and MVP5180-91 representing HIV-1 group O formed a panel that was initially used for the optimization process of the primers and the assay system. Cell culture supernatants MVP11971-87 and MVP438-01 representing HIV-2 were used to test the specificity of the assay, whereas ultra pure water (H_2O) and 0.9% sodium chloride (NaCl) were used as negative controls. Total RNA was extracted from these cell culture supernatants using an automated system, Roche MagNAPure (Roche Diagnostics, Mannheim, Germany), according to manufacturer's instructions. Ten fold dilutions were made from the extracted RNA materials which formed a quantification panel ranging from 2.1×10^1 to 2.1×10^6 copies/mL. Loopamp® RNA Amplification kit and Loopamp® fluorescence detection dye (Eiken Chemical Co., Ltd, Tochigi, Japan) and MAST Isoplex™ RNA Amplification kit (Mast Diagnostica, Reinfeld, Germany) were used for LAMP amplifications and detections. Restriction enzymes (New England Biolabs Inc. Frankfurt, Germany), Biozym LE agarose (Biozym Scientific, Oldendorf, Germany) and DNA marker VIII (Roche Diagnostics, Mannheim, Germany) were used for amplification product characterization.

3.2.1.2 Design and synthesis of LAMP primers

Four sets of primers required for the LAMP reaction were designed recognizing six arbitrary regions of the target sequences of the *gag*-p24 gene of HIV-1 BaL sequence, Gene Bank accession number AY713409 (Curtis et al., 2008) and the *pol*-integrase gene, accession number K02013 (Hosaka et al., 2009), further the U3 start sequence of the LTR and the N-terminal of gp-41 of the *env* gene both from the HXB2 reference genome sequence. Loop primers (LF and LB) were included to accelerate the LAMP reaction. The new primers were designed using PrimerExplorer V3 software (Eiken Chemical Co. Ltd. website (http://primerexplorer.jp/e/)) and LAMP designer (Premier biosoft.com). All primers were synthesized by Ella Biotech, Munich, Germany.

Primer set	Primer name	Sequence (5´to 3´)
HiP	F3 **(1311-1329)**	ATTATCAGAAGGAGCCACC
	B3 **(1515←1535)**[RC]	CATCCTATTTGTTCCTGAAGG
	FIP	**(1340←1361)**[RC] CAGCTTCCTCATTGATGGTTTCTTT - **(1393-1417)** TTAACACCATGCTAAACACAGT
	BIP	**(1455-1478)** TGTTGCACCAGGCCAGATAATTTT - **(1494←1514)**[RC] GTACTGGTAGTTCCTGCTATG
	LF **(1370←1394)**[RC]	TTTAACATTTGCATGGCTGCTTGAT
	LB **(1475-1493)**	GAGATCCAAGGGGAAGTGA
HiInteg	F3 **(4721-4743)**	GGTAAGAGATCAGGCTGAACATC
	B3 **(4926←4945)**[RC]	GCTGGTCCTTTCCAAAGTGG
	FIP	**(4747←4766)**[RC] CCCCAATCCCCCCTTTTCTT - **(4787-4806)** AGACAGCAGTACAAATGGCA
	BIP	**(4812-4835)** AGTGCAGGGGAAAGAATAGTAGAC - **(4900←4921)**[RC] CTGCTGTCCCTGTAATAAACCC
	LF	TTAAAATTGTGGATGAAT
	LB	GCAACAGACATACAAACTAAAG
HiLtr	F3 **(29-50)**	ACAAGATATCCTTGATCTGTGG
	B3 **(319←337)**[RC]	CGATGTCAGCAGTTCTTGT
	FIP	**(158←179)**[RC] GGCTTCTTCTAACTTCTCTGGC – **(106-126)** GGATCAGATATCCACTGACCT
	BIP	**(190-210)** AGAACACCAGCTTGTTACACCA - **(245←264)**[RC] CCTCCACTCTAACACTTCT
	LF **(127←147)**[RC]	CTAGCTTGAAGCACCATCCAA
	LB **(215-232)**	GAGCCTGCATGGAATGGA
HiGp	F3 **(8740-8755)**	GAG CTA TTC GCC ACA TAC C
	B3 **(8964←8979)**[RC]	CTC TTG TGC TTC TAG CCA G
	FIP	**(8846←8866)**[RC] CAGCTCGTCTCATTCTTTCCC – **(8786-8805)** TTTGCTATAAGATGGGTGGC
	BIP	**(8874-8891)** AGCAGATAGGGTGGGAGC – **(8920←8941)**[RC] CTGTATTGCTACTTGTGATTGC
	LF **(8819←8835)**[RC]	AGG CCA TCC AAT CAC ACT AC
	LB **(8895-8909)**	AGC ATC TCG AGA CCT GGA

Table 1. List of primers and their target positions within the HIV genome. **Hip** set of primers are designed to target the protein (p24) of the *gag* gene. **HiInteg** primers target the integrase of the *pol* gene. **HiLtr** is designed from the U3 start region of the long terminal repeat (LTR) and **HiGp** was designed to target the N-terminal part of the glycoprotein (gp41) of the *env* gene. Reverse complement primers (RC -in red color). FIP and BIP sequences combine 2 sequences (F1c+F2 and B2+B1c). *F3 – Forward primer; B3 – Backward primer; FIP – Forward Inner Primer; BIP – Backward Inner Primer; LF – Loop Forward; LB – Loop Backward.*

3.2.1.3 Testing of primers

Binding stringency of LAMP primers was tested on both normal rt-PCR using F3 and B3 primers and rt-LAMP assay.

3.2.1.3.1 rt-PCR

Reverse transcription of HIV template RNA was done before PCR amplification using 2 µL B3 primers (10 µM each), 4 µL 5x buffer (100 µM Tris-HCL, 250 µM KCL, pH 8.4), 2 µL dithiothreitol (DTT) (0.1 M), 2 µL desoxynucleotide triphosphates (dNTP) (5 mM) and 40U reverse transcriptase enzyme (cat. no. 18064-014, Life Technologies, Germany). 10 µL of the mix was incubated with 10 µL of the samples at 45ºC for one hour. PCR was performed with 2U Taq polymerase enzyme (cat. no. N 808-0152, Life Technologies, Germany) in a master mix of dH_2O, dNTP (5 mM), 10x buffer (200 mM Tris-HCL (pH 8.4), 500 µL KCL), Primer 1 (F3) and Primer 2 (B3) each 10 µM. 43 µL of the master mix was incubated with 7 µL of cDNA template and run in the first PCR amplification. The second PCR was performed with 2U Taq polymerase enzyme (cat. no. 10342-020, Life technologies, Darmstadt, Germany) in a master mix containing dH_2O, dNTP (5 mM), 10x Taq polymerase buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), Primer 3 (FIP) and Primer 4 (BIP). A total of 48 µL of the master mix was incubated and run in the second PCR amplification after 2 µL of the amplification product (amplicon) of the first PCR was added.

3.2.1.3.2 rt-LAMP amplification

The rt-LAMP reaction was carried out using the MAST Isoplex™ RNA Amplification kit (Mast Diagnostica, Reinfeld, Germany) containing 8 U/µL Bst polymerase and 20 U/µL reverse transcriptase enzymes, 3 mM hydroxynaphthol blue (HNB) dye, a fluorescence dye (V13), water and an already constituted 2x reaction mix (RM). The reaction mix contained dNTPs (2.8 mM), KCl (20 mM), $(NH_4)_2SO_4$ (20 mM), Tris buffer (40 mM, pH 8.8), betaine (1600 mM) and $MgSO_4$ (16 mM). Briefly, 12.5 µL of the reaction mix, 1 µL of the fluorescence or colour detection dye, 1 µL of Bst polymerase enzyme, 0.5 µL reverse transcriptase, water and different concentrations and volumes of primers were mixed together in a 25 µL reaction. This was then set at an optimal temperature of 63°C for 1 hour. For visualization under UV light, 1 µL of SYBR green fluorescence detection dye (Eiken Chemical Co., Ltd, Tochigi, Japan) was used instead of the detection dyes supplied in the amplification kit. Sample volume added was dependent on the concentration and the volumes of the primers during this initial stage.

3.2.1.4 Optimization of rt-LAMP reaction conditions

Primers were first evaluated and tested for selection of the best suitable primers. Varying concentrations and volumes of the primers and templates of the cell culture dilution panels were tested and time to detection recorded. The primer concentrations included; FIP (40 pmol and 50 pmol), BIP (40 pmol and 50 pmol), F3 (5 pmol), B3 (5 pmol), LF (20 pmol and 25 pmol), LB (20 pmol and 25 pmol). The primer volumes also varied between 2 µL and 3 µL. The amount of the target

RNA template ranged between 3 µL to 5 µL. The reaction mixture was incubated at 63°C for 60 min in a LoopAmp real-time turbidimeter (LA-200m; Teramecs, Kyoto, Japan). The turbidimeter measures the amount of turbidity as a result of magnesium pyrophosphate ($Mg_2P_2O_7$) deposits generated during the amplification process. With the aid of a special fluorescence dye (V13) a real time measurement was obtained, where a threshold value (Abs) equivalent of an optical density above 0.1 indicated a positive detection (Figure 3 (3e)). To assess the specificity of non specific products, the amplicons from rt-LAMP were run on a 2% agarose gel electrophoresis (Biozym Scientific, Oldendorf, Germany). The visual inspection of the processed amplification was also ascertained using HNB detection dye, through inspection of visual turbidity due to magnesium pyrophosphate ($Mg_2P_2O_7$) deposits or under UV light using a fluorescent detection SYBR green intercalating dye (Eiken Chemical Co., Ltd, Tochigi, Japan).

3.2.1.5 Analytical sensitivity and specificity of the rt-LAMP

3.2.1.5.1 Sensitivity

Serial dilutions of cell culture supernatant panels of IIIB and MVP899-87 viruses ranging from $0.12x10^1$ to $2.9x10^6$ copies/mL (Figure 12) were tested between 4 to 18 times for individual samples in the assay and the results recorded. An in-house software (CERES) developed by the Virus Diagnostic Department of the Max von Pettenkofer-Institute was used to calculate the lower limit of detection (LLOD) and the lower limit of quantification for the LAMP assay. Viral loads were compared to those of an established Abbott Real Time HIV-1 commercial assay (Abbott m2000rt system) for HIV-1 nucleic acid amplification and quantification.

3.2.1.5.2 Specificity

Purified RNA templates from cell culture supernatants, MVP11971-87 and MVP438-01 representing HIV-2 were each tested 14 times and the results recorded. LAMP amplicons that showed unspecific amplification ("smear") by gel electrophoresis were digested in a Restriction Fragment Length Polymorphism (RFLP) assay using a set of restriction enzymes. Based on sequence alignment of HXB2 (IIIB virus), restriction enzymes were designed by online restriction tools (*RestrictionMapper* version 3.0; http://www.restrictionmapper.org/ and *NEBcutter* Version 2.0; New England Biolabs Inc.). The following restriction enzymes were selected and tested.

Enzyme	Restriction sequence	Restriction product length	LAMP product length	Catalogue number	Primer set
BsmAI	5'… G T C T C N↓NNNN…..3' 3'… C A G A G N NNNN↑…..5'	205 bp	225 bp	R 0529S	HiP
BsrGI	5'…. T↓G T A C A……3' 3'…. A C A T G↑T……5'	193 bp	203 bp	R0575	HiInteg

Table 2. Restriction enzymes and their target primer sets. The list shows restriction enzymes for amplicons of p24 (HiP) – BsmAI and integrase (HiInteg) – BsrGI target primers and lengths for their target products.

3.2.2 Processing and testing of clinical samples by LAMP assay

Clinical samples were received either as whole blood in EDTA or as plasma samples (mainly for archived samples) and processed as described in Figure 3. Upon reception, nucleic acid was either extracted directly from whole blood (**A**) or whole blood was centrifuged to separate plasma (**B**) from which the nucleic acid was extracted before the LAMP analysis (**Bi**). Non-processed samples were also analyzed directly by the LAMP assay after pre-heating at 100°C for 5 minutes or after treatment with lysis buffer (**Bii**). Dry blood or dry plasma spots were processed and stored at -80°C (**C**).

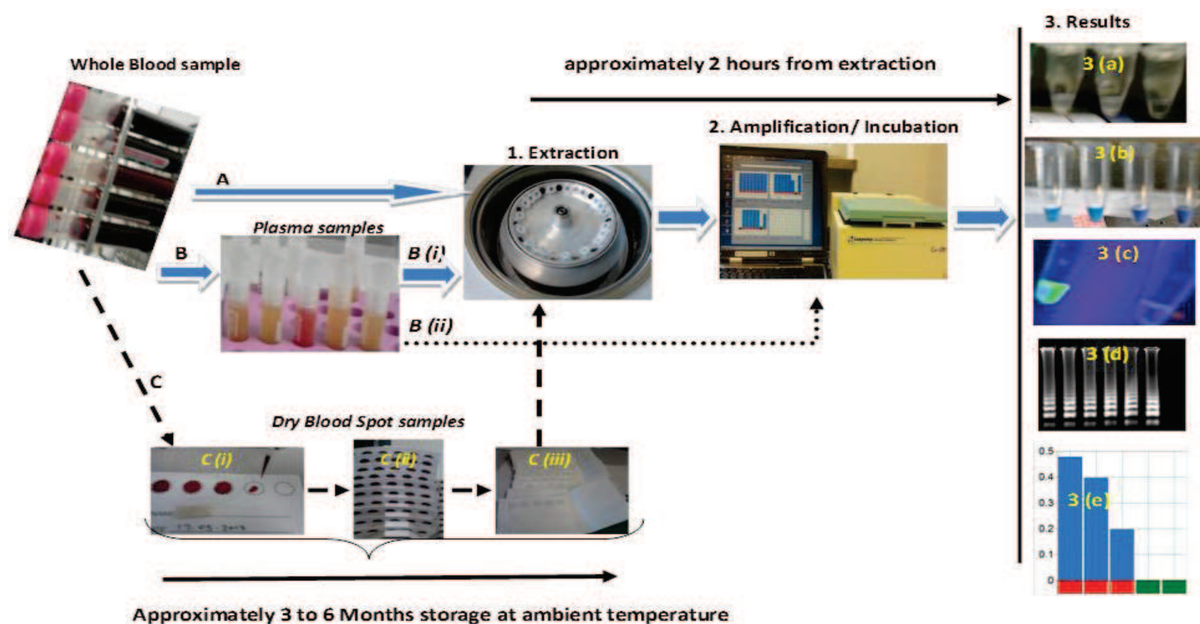


Figure 3. Summary scheme of laboratory procedures. In (A) the samples were processed for nucleic acid extraction (1) directly from whole blood samples and incubated for amplification. In (B) plasma was obtained from whole blood then either nucleic acid extracted before amplification (Bi) or the plasma was amplified directly after pre-heating at 100°C for 5 minutes or treatment with lysis buffer (Bii). In (C) whole blood was spotted on FTA cards (Ci) then dried (Cii) and packaged in special envelopes with desiccants (Ciii) and stored either at ambient temperature or at -80°C for 4 to 6 months before shipment. The time duration from extraction to final results was estimated at 2 hours and the results were read as shown in 3a, 3b, 3c, 3d and 3e.

3.2.2.1 Preparation of dry blood spot or dry plasma samples

As shown in C (Figure 3) DBS samples were prepared by spotting 70 µL of whole blood or plasma onto each of the 5 half-inch circles on the Whatman 903 neonatal blood FTA collection cards (Lot. 6273207/51, GE Healthcare Europe, Freiburg Germany). The blood spots were then left to dry on a Whatman card drying rack overnight, before the cards were packed into gas impermeable bags with desiccant packs and humidity indicator cards. The dry spots were stored at ambient temperature or at -80°C if long storage of more than 6 months was anticipated.

3.2.2.2 Procedures for sample manipulation

The following steps as already shown in Figure 3 were applied during the laboratory procedures.

3.2.2.2.1 Manual extraction of nucleic acid

3.2.2.2.1.1 Extraction from whole blood and plasma

Manual extraction (Figure 3, step 1) was done on all samples using the High Pure Viral Nucleic Acid Kit (cat. no. 11858874001) from Roche Diagnostics (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. Briefly, a volume of 200 µL of each sample was mixed with 200 µL binding buffer supplemented with poly (A) and 50 µL (50 mg/µL) of proteinase K and incubated at 72°C for 10 minutes. After incubation, a further volume of 100 µL of the binding buffer was added to each sample, mixed, and the sample pipetted to High Pure Filter tubes, connected to the connection tube. This complex was then centrifuged at 8,000g for 1 minute, before a volume of 500 µL of the inhibitor removal buffer was added to the filter tubes and centrifuged for 1 minute at 8,000g. Two steps of washing were done using 450 µL wash buffer in each case followed by centrifugations at 8,000g and 13,500g for 1 minute and 30 seconds respectively in the first and second washing steps. This procedure was then followed by an elution step, where RNA was eluted in 50 µL elution buffer.

3.2.2.2.1.2 Extraction from dry blood spots

One spot was punched out from the cards and incubated in 1,000 µL lysis buffer obtained from the Abbott sample preparation system kit (Promega, Madison, U.S.A). The incubation was done at 55°C for 10 minutes shaking at 550 rpm in a thermomixer heating block. The sample material was centrifuged at 8,000g for 3 minutes, before the supernatant was transferred into a new tube. Nucleic acid extraction was performed using the High Pure Viral Nucleic Acid extraction kit as described above.

3.2.2.2.2 RNA linearization

Before amplification HIV RNA was linearized by heating the isolated RNA suspension to 72°C for 10 minutes. This process was followed by immediate cooling on ice for at least 5 minutes before the samples were processed for amplification. Alternatively, the linearized RNA was stored at -20°C if delayed nucleic acid amplification process was anticipated.

3.2.2.2.3 Treatment of samples of various categories

i. **Nucleic acid extracted plasmas:** Samples were treated as described in chapter 3.2.2.2.1.1 and then used for comparisons during evaluations in steps iii and iv.

ii. **Nucleic acid extracted DBS:** Samples were treated as described in chapter 3.2.2.2.1.2 and used for comparisons during evaluations in steps iii and iv.

iii. **Heated non-extracted plasma samples:** These samples were first mixed with RNAse free water in the ratio of 1:3 to avoid clotting and then subjected to 99°C for 7 minutes on a heating block, thereafter 5.5 µL and 9.5 µL were separately used in the LAMP reaction.

iv. **non-extracted plasma samples treated with a lysis buffer:** These samples were first diluted in a lysis buffer supplied for extraction in the ratio 1:3 to make a starting volume of 200 µL. Subsequently a volume of 5.5 µL of the mixture was used in LAMP assay evaluation.

3.2.2.2.4 rt-LAMP amplification process

As shown in step 2 (Figure 3), amplification of the linearized products was done using the MAST Isoplex™ RNA Amplification kit (Mast Diagnostica, Reinfeld, Germany). Briefly, the final reaction volume of 25 µL contained: a primer solution of 0.2 µM of F3 and B3, 1.6 µM FIP and BIP and 0.8 µM of LF and LB primers; 12.5 µL reaction mix containing dNTPs (2.8 mM), KCl (20 mM), $(NH_4)_2SO_4$ (20 mM), Tris buffer (40 mM, pH 8.8), betaine (1600 mM) and $MgSO_4$ (16 mM); 1 µL of 3 mM HNB or fluorescent (V13) detection dyes; 8 U of Bst polymerase; 10 U of reverse transcriptase and 2.5 µL of water. These components were mixed with 5 µL of the templates, incubated at 63°C for 1 hour in a real time turbidimeter (LA-200m; Teramecs, Kyoto, Japan) and the time to threshold detection recorded. The time to threshold detection was later used to calculate the estimation algorithm for viral load. Incubation was also done on a heating block set at 63°C for 1 hour and results observed visually by turbidity or visual colour change.

3.2.2.2.5 Detection of LAMP products and generation of results

The products generated after rt-LAMP amplification process were detected as desribed in step 3 (Figure 3) by visual examination of turbidity (3a), colour change due to HNB dye (3b), fluorescence

detection by SYBR green dye (3c), gel electrophoresis (3d), and real time detection using the LAMP turbidimeter (3e).

3.2.3 Automated extraction and quantification of viral load by Abbott Real Time HIV-1 assay

Processing and quantification of samples was first done by the Abbott Real Time HIV-1 assay as described by the manufacturer http://www.abbottmolecular.com/static/cms_workspace/pdfs/US/51-602146R6.pdf. Abbott Real Time HIV-1 is a complete assay which utilizes an automatic extraction of samples by use of Abbott (m2000sp) systems followed by amplification and quantification using Abbott (m2000rt). The starting sample volume for automatic nucleic acid extraction was 1,000 µL and where the appropriate volume could not be attained, samples were first diluted in 0.9% NaCl and then used for analysis.

3.2.4 Nucleic acid sequencing

The sequencing process involved 3 main phases and was performed according to manufacturer´s protocol.https://www.beckmancoulter.com/wsrportal/bibliography?docname=Protocol_000387v001.pdf. (Agencourt Bioscience Corporation, Beverly, MA, United States). This sequencing method is based on the technique developed by Sanger (Sanger et al., 1977), also known as Sanger sequencing. It mainly involves selective incorporation of chain-terminating di-deoxynucleotides by DNA polymerase enzyme during in vitro DNA replication. The di-deoxynucleotide-triphosphates (ddNTPs) are responsible for terminating the strand synthesis.

3.2.4.1 Purification of PCR products

This phase was performed using the Agencourt® AMPure® XP PCR purification system which utilizes a solid-phase paramagnetic bead technology for high-throughput purification of PCR amplicons. By using an optimized buffer, PCR amplification products of 100 bp or larger are selectively bound to paramagnetic beads. Excess primers, nucleotides, salts, and enzymes are then removed through a washing step, resulting in purified PCR products.

In summary, products of PCR amplification were bound to magnetic beads by adding and mixing 72 µL of AMPure and 40 µL PCR reaction products followed by incubation for 7 minutes, and separated on a magnetic plate (SPRIPlate 96R). The beads were then washed using 200 µL of freshly prepared 70% ethanol to remove excess primers, nucleotides, salts, enzymes other contaminants before the products were finally eluted in 40 µL elution solution (dH_2O).

Quantity of recovered products was estimated reading the optical signals generated by the DNA using PicoGreen® intercalating dye (Cat.P11496, Life Technologies, Darmstadt, Germany)

3.2.4.2 Pre-sequencing product preparation and sequencing

Before product preparation and sequencing, a sequence reaction mixture was prepared in which eluted DNA aliquots were diluted in dH_2O to set up 5.5 µL of the mixture. A sequence reaction consisting of 5.5 µL reaction mixture, 0.5 µL of 10 pmol/µL primer (targeting the integrase gene) and 4.0 µL of Quick Start Master was then set to a 30 cycle sequence reaction PCR.

Pre-sequencing product preparation and sequencing was then performed using the CleanSEQ® Dye-Terminator Removal method as described by Agencourt®. This is a 3-step Solid Phase Reversible Immobilization (SPRI) magnetic bead-based sequencing purification system which is flexible, simple and can be performed directly in the thermal cycling plate without centrifugation or filtration.

In the first step 10 µL of CleanSEQ and 45 µL of 85% freshly prepared ethanol were added to the sequencing extension products generated during the cycle sequencing, mixed and incubated on a magnetic plate in order to bind the products to the magnetic beads. The bound products were then washed using 100 µL of 70% ethanol to remove unincorporated dyes, nucleotides, salts, and other contaminants. The products were then eluted in 40 µL aqueous SLS elution buffer, before a drop of oil was added to the wells and the plate loaded into a CEQ 8,800 Beckman device for sequencing.

3.2.4.3 Editing of sequences and HIV subtyping

The nucleic acid sequence results generated were edited and finally aligned using a Bioedit software (Hall, 1999) (Ibis Biosciences - Carlsbad, CA,USA). Sequences were subtyped and assigned using Context based modelling for expeditious typing (COMET HIV-1), an online subtyping tool (http://comet.retrovirology.lu/).

Chapter 4

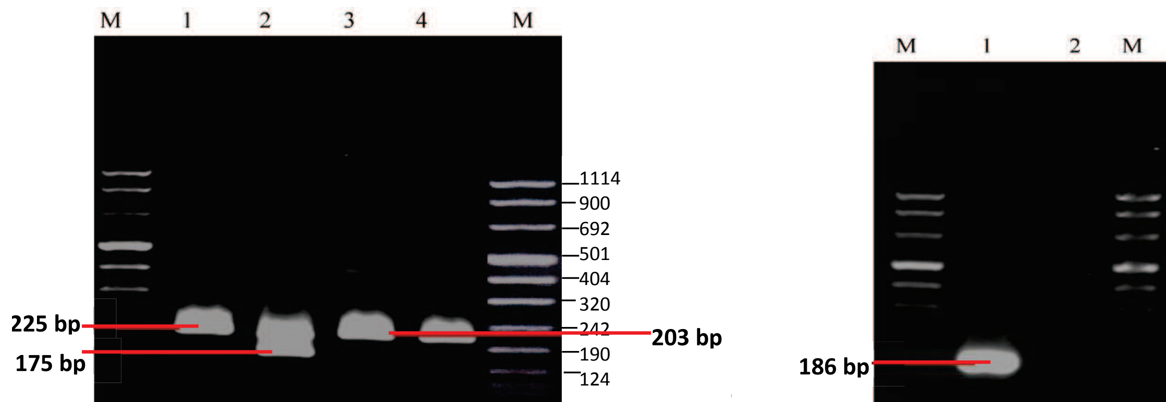
4 Results

4.1 Testing efficacy of primers

4.1.1 rt-PCR

4.1.1.1 Testing primers by rt-PCR

The four sets (HiP, HiLtr, HiInteg and HiGp) of primers designed, as described in chapter 3, were first subjected to conventional rt-PCR using F3, B3, FIP and BIP pairs in each case. The results obtained showed amplification of the expected targets at the calculated fragment lengths of 225 bp (HiP), 175 bp (HiLtr), 203 bp (HiInteg) and 186 bp (HiGp) as shown in gel electrophoresis photos of analyzed PCR products (Figure 4).



Gel 1a shows amplification products by rt-PCR for HiP, HiLtr and HiInteg sets of primers respectively using culture supernatants of MVP899-87 viruses. **M:** molecular weight marker VIII, **1:** HiP primers, **2:** HiLtr **3:** HiInteg primers, **4:** In-house (integrase target primer) positive control.

Gel 1b shows amplification product by rt-PCR at position 186 bp for HiGP set of primers on culture supernatant of MVP899-87 **M:** molecular weight marker, **1:** positive sample, **2:** negative control (water)

Figure 4. Products of rt-PCR amplification generated by LAMP designed primers as detected by agarose gel electrophoresis in the presence of ethidium bromide. Results show clear bands at the calculated fragment legnths for each primer set. There was no background band formation despite intensive staining which indicates high efficiency with the primers. The primers were selected to target 4 conserved regions of the HIV genome, namely the LTR, *gag, pol,* and the *env*. The results show that the primers were suitable for use in rt-PCR.

4.1.2 rT-LAMP

4.1.2.1 Optimization of primers and reaction conditions

Optimization of primers and reaction conditions was performed for three (HiP, HiInteg and HiLtr) of the total four sets of primers. Variations in concentrations and volumes were done to compare and select favorable attributes of performance of the LAMP assay. Incubations were done on a real time

turbidimeter and a manual heating block and detections were performed using detection dyes, (fluorochrome dye (FD) – for turbidimetry and HNB for visual colour change) both supplied by Mast diagnostics (Mast Diagnostica, Reinfeld, Germany).

Real-time monitoring of LAMP amplification is mainly achieved through spectrophotometric signals measured by real-time turbidimetry. The turbidimeter records the turbidity in the form of optical density (OD) values recorded every 6 seconds at 400 nm (Parida *et al.,* 2008). LAMP reaction results in the production of large amounts of pyrophosphate by-products which react with magnesium ions leading to the formation of a white precipitate of magnesium pyrophosphate.

The effect of variation of volumes from a stock solution comprising of 0.2 µM F3 and B3, 1.6 µM FIP and BIP, 0.8 µM LF and LB primer concentrations was evaluated. When a volume of 2 µL of primer was used in a 25 µL total reaction volume, 2 samples of high virus concentrations were not detected by HiP set of primers either by turbidimetry, visual turbidity or visual colour change, whereas HiInteg and HiLtr detected these 2 samples using the same primer volume by all the mentioned detection methods (Table 3). Gel electrophoresis results however showed "smears" for the 2 samples tested by HiP set of primers (Figure not shown).

When a volume of 2.5 µL was used, HiP set of primers showed positive detection (27 – 35 minutes) by turbidimetry, for the viral load of 2.1×10^5 copies/mL. Viral concentration approximately 2.1×10^2 copies/mL recorded late detection (> 45 minutes) by the same set of primers. The HiInteg primer set recorded early detections (26 - 33 minutes), with the same primer volume, while the HiLtr primer set showed late detections (34 - 39 minutes) for virus concentration of 2.1×10^5 copies/mL tested in triplicates. The lowest virus concentration detected by the HiInteg set of primers was 2.1×10^2 copies/mL compared to that detected by HiLtr (2.1×10^4 copies/mL).

There was however lack of consistency in results generated by the HiInteg and HiLtr sets of primers when the volume of primers was increased to 3 µL as shown in test 3 and 4 (Table 3). An increased concentration of the inner primers (FIP and BIP) and the loop primers (LF and LB) did not yield any significant improvement of detection for HiP and HiInteg set of primers as shown in test 4 (dotted square) in Table 3. Although the results shown in Table 3 were each generated with a sample volume of 5 µL in a 25 µL total reaction, it was realized that similar results were obtained when 3 µL of the samples were used. However, when 3 µL was used, only samples of viral loads $>2.1 \times 10^3$ copies/mL were easily detected by the HiP set of primers whereas HiInteg produced inconsistent results which were considered unreliable.

Primer set	Virus concentration	Test 1 (2 µL primers) Tt value	Vis. Turb	Gel results	Colour change	Test 2 (2.5 µL primers) Tt value	Vis. Turb	Gel results	Colour change	Test 3 (3 µL primers) Tt value	Vis. Turb	Gel results	Colour change	Test 4 (2 µM FIP/BIP & 1 µM LF/LB) Tt value	Vis. Turb	Gel results	Colour change
HiP	$2.1x10^5$	-	-	(+/-)	-	27.24- 35.42	+	+	+	26.48- 28.48	+	+	+	31.24	+	+	+
	$2.1x10^4$	-	-	(+/-)	-	33.06- 38.12	+	+	+	30.24- 31.24	+	+	+	41.54	+	+	+
	$2.1x10^3$	-	-	-	-	35.24- 40.54	+	+	+	48	+	+	+	28.36	+	+	+
	$2.1x10^2$	-	-	-	-	45.54- 49.04	+	+	+	-	-	-	-	55.54- 56.06	+	+	+
	$2.1x10^1$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.1	-	-	-	-	39	+	+	+	-	-	-	-	-	-	-	-
	NC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HiInteg	$2.1x10^5$	23.32- 32.06	+	+	+	26.42- 33.36	+	+	+	-	-	-	-	39.30- 41.00	+	+	+
	$2.1x10^4$	26.06- 32.24	+	+	+	33.12- 39.24	+	+	+	38.14- 48.06	+	+	+	(+/-)	+	+	+
	$2.1x10^3$	39.54-43.12	+	+	+	39.42- 50.02	+	+	+	-	-	-	-	48.32-50.54	+	+	+
	$2.1x10^2$	49.06-53.06	+	+	+	41.54	+	+	+	-	-	-	-	-	-	-	-
	$2.1x10^1$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HiLtr	$2.1x10^5$	34.12- 52.12	+	+	+	34.06- 39.42	+	+	+	45.02- 49.06							
	$2.1x10^4$	47.32- 59.24	+	+	+	40.54- 48.00	+	+	+	-							
	$2.1x10^3$	-	-	-	-	-	-	-	-	-							
	$2.1x10^2$	-	-	-	-	-	-	-	-	-							
	$2.1x10^1$	-	-	-	-	-	-	-	-	-							
	2.1	-	-	-	-	-	-	-	-	-							
	NC	-	-	-	-	-	-	-	-	-							

Table 3. Optimization of the primer concentration for LAMP analysis. The table shows a summary of the effects of variation of primer volume from a stock of 0.2 µM (F3 and B3), 1.6 µM (FIP and BIP), 0.8 µM (LF and LB) primer concentrations on different concentrations of a dilution panel of HIV-1 M:B IIIB virus. The optimum primer volume of 2.5 µL in a total reaction volume of 25 µL (shown in the square box) was achieved for LAMP assay (Test 2). An increase in the primer concentrations for the inner primers (FIP/BIP) and the loop primers (LF/LB) as shown in the square dotted box, did not yield any improved detection compared to when 2.5 µL from the concentration of primer stock solution was used. Indeterminate results (±) shown in the table as (+/-) was indicated by a "***smear***" by gel electrophoresis and ***"no Tt value"*** for real time turbidimeter, **Tt value:** Time to threshold detection (given in minutes), **Vis Turb:** visual turbidity, **NC :** Negative control. Positive detections are shown by a plus (+) sign, whereas negative results are shown by a dash (-).

4.1.2.2 Restriction enzyme digestion of rt-LAMP products

In order to confirm the specificity of the LAMP products shown by the ladder like bands generated by gel electrophoresis, these amplicons which had been generated by HiP and HiInteg sets of primers were digested using the set of restriction enzymes described in chapter 3 (Table 2). The aim was to generate single bands of the expected sizes. The results obtained showed single bands of the calculated sizes for the different enzymes (Figure 5) an indication of specificity of primer binding to their targets.

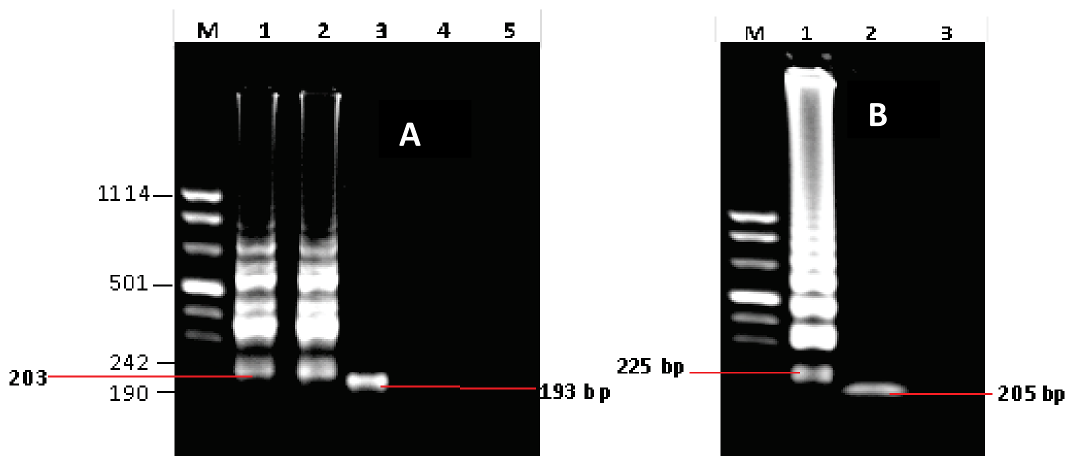


Figure 5. Restriction digestion of rt-LAMP amplicons generated by LAMP designed primers. Agerose gel photo A shows a single restriction band at 193 bp generated by ***BsrGI*** enzyme for amplicons of HiInteg set of primers. Agarose gel photo B shows a single restriction band generated by ***BsmAI*** enzyme at 205 bp for amplicons of HiP set of primers. **Agarose gel photo A:** *M* **-** DNA marker VIII, *track 1 and 2* – LAMP amplicon, *track 3* -restriction band, *track 4* and 5 – Negative control. **Agarose gel photo B:** *M* **–** DNA molecular weight marker VIII, *track 1* – LAMP amplicon, *track 2* – restriction band, track 3 – negative control. Restriction bands generated at expected fragment lengths (Table 2).

4.1.2.3 Testing and detection of LAMP products with cell culture supernatants

Testing of LAMP after optimization was done on cell culture supernatants of IIIB and MVP899-87 viruses. Figure 6 shows detection methods used for interpreting the results, mainly visual turbidity, visual colour change, fluorescence detection, gel electrophoresis and real time turbidimetry.

Figure 6a. Tubes 1 and 2 show positive results by turbidity. Tube 3 shows negative non-turbid result

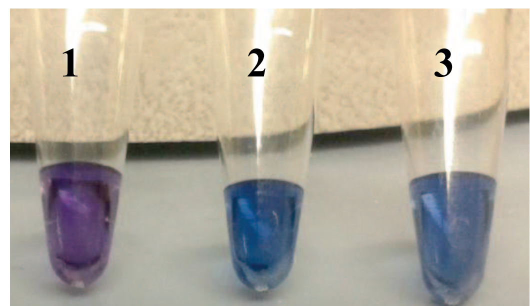


Figure 6b. Results by visual color detection. Tubes 1 purple negative result. Tubes 2 and 3 pale blue positive results by HNB color detection dye.

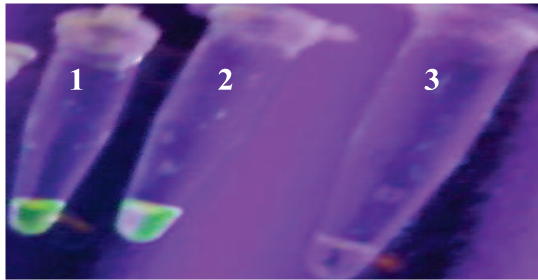


Figure 6c. Tubes 1 and 2 show positive results by SYBR green detection dye. Tube 3 shows a negative result.

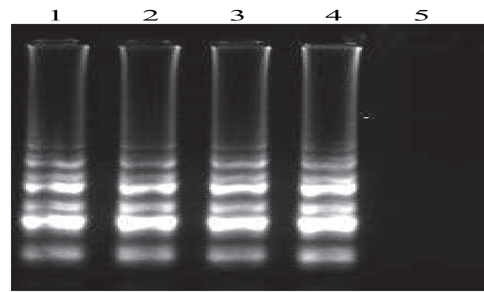


Figure 6d. Tracks 1 to 4 show ladder-like bands of LAMP amplification on agarose gel. Track 5 shows a negative control result.

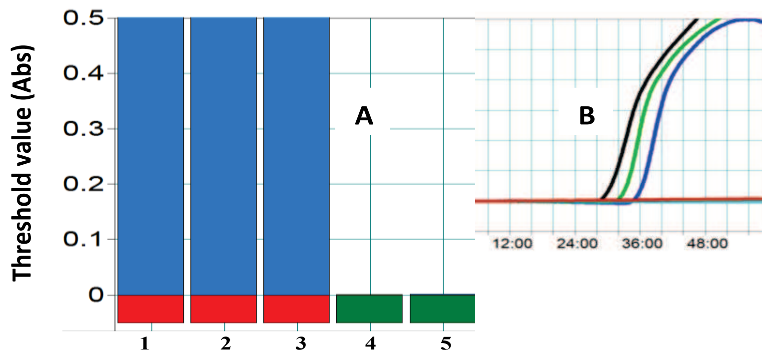


Figure 6e. Read outs of real time turbidimetry results. Wells 1 to 3 in figure A show blue bars with red origins indicating positive results, while wells 4 and 5 remain green indicating negative results. Figure B shows real time amplification results. In both figures, vertical line represents the threshold value (Abs) generated as a result of optical density (OD) signals while in figure B, the horizontal line represents time to threshold detection in minutes. The threshold value (optical density) of 0.1 was the value above which the positive signals were generated by LAMP turbidimeter

Figure 6. Detection methods used during LAMP evaluation. The figure shows various detection methods that were used for LAMP. **6a:** turbidity due to presence of magnesium pyrophosphate. **6b and 6c:** visual colour change by hydroxynaphthol blue (HNB) and fluorescent (SYBR green) dyes. **6d:** gel electrophoresis showing ladder-like bands which are typical for LAMP amplicons. **6e:** turbidimetric detection using real time turbidimeter

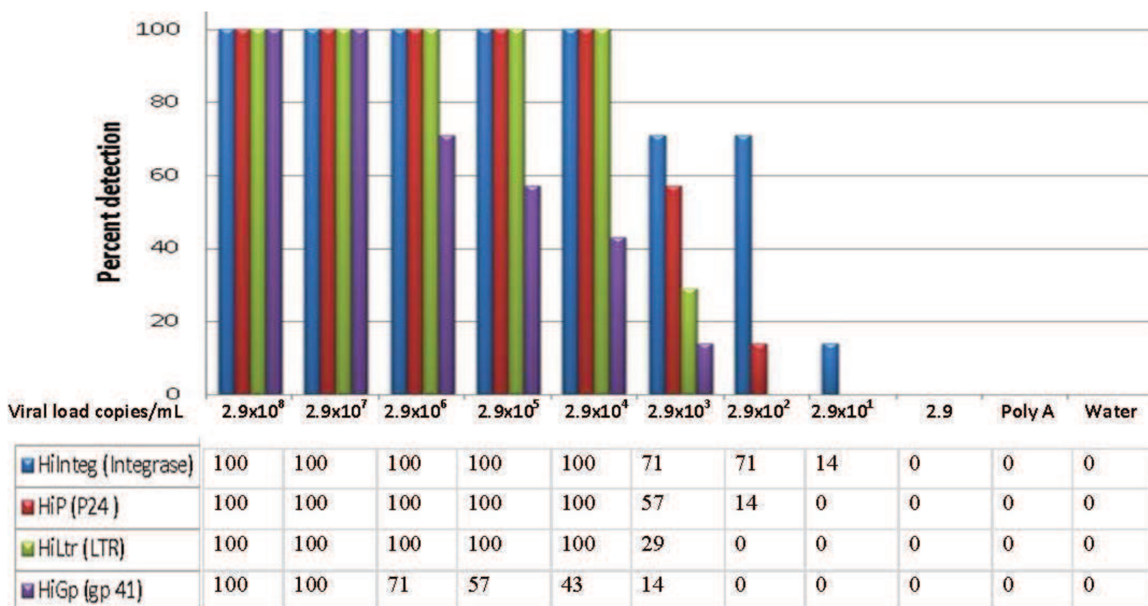
4.1.2.4 Prevention of sample cross contamination

During the first stages of this study, cross contamination with amplified products was realized, when a series of negative samples also tested positive. Source of contamination was traced to some of the tube racks which were returned to the lab after opening the LAMP amplification tubes for analysis by gel electrophoresis. Apart from changing the rooms and replacing all the racks, pipettes and other containers, a series of precautions were introduced for subsequent analysis. These included the recommended 3 room strategy: extraction was done in the first room, master-mix was prepared in the second room in one clean bench and a second bench was used during sample loading and a third room was used for real time amplification. Upon completion of LAMP reaction, all tubes were discarded without opening.

4.1.2.5 Testing and evaluation of analytical parameters of LAMP primer sets.

4.1.2.5.1 Sensitivity across a dilution panel

Different dilutions of MVP899-87 virus ranging from 2.9 to $2.9x10^8$ copies/mL were tested 7 times and the results recorded either as positive or negative. Comparisons in primer performance showed that the HiInteg set exhibited the highest sensitivity among the panel of HIV-1 M subtype B (Figure 7). This set of primers detected up to 14% of samples with a concentration of $2.9x10^1$ copies/mL. Primer set HiP also exhibited a high sensitivity presenting a detection limit of $2.9x10^2$ copies/mL. All primer sets, except for primers HiGp detected all samples $\geq 2.9x10^5$ copies/mL as quantified by Abbott m2000rt system.



Viral load copies/mL	$2.9x10^8$	$2.9x10^7$	$2.9x10^6$	$2.9x10^5$	$2.9x10^4$	$2.9x10^3$	$2.9x10^2$	$2.9x10^1$	2.9	Poly A	Water
HiInteg (Integrase)	100	100	100	100	100	71	71	14	0	0	0
HiP (P24)	100	100	100	100	100	57	14	0	0	0	0
HiLtr (LTR)	100	100	100	100	100	29	0	0	0	0	0
HiGp (gp 41)	100	100	71	57	43	14	0	0	0	0	0

Figure 7. Evaluation of analytical sensitivity and rate of detection of LAMP primers for the cell culture supernatant dilution panel. Results show the percentage detection rate (n=7) of a dilution panel of the MVP899-87 virus for different primer sets detected by real time turbidimetry and confirmed by gel electrophoresis. Best performance within this range of virus concentrations is exhibited by HiInteg and HiP sets of primers which detected ≥ $2.9x10^1$ and $2.9x10^2$ copies/mL respectively. Primer sets HiLtr and HiGp both showed lower sensitivities for these samples.

4.1.2.5.2 Linearity across the primer sets

Analysis of the linearity of the amplification reaction was performed with decreasing concentrations of the MVP899-87 sample. The results in Figure 8 depict an inverse positive linear correlation between concentration and time for all primer sets. Samples with higher viral loads tested with the HiGp set of primers showed the earliest time to threshold detection at 13 minutes followed by HiInteg and HiP both at 20 minutes, while the earliest time to detection realized with HiLtr set of primers was 30 minutes for the same high viral concentrations.

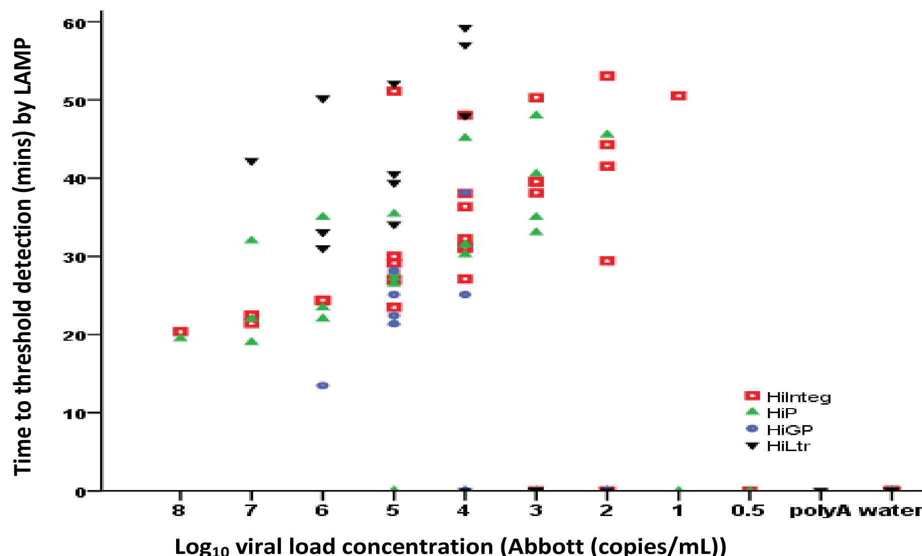


Figure 8. Results of a dilution panel of MVP899-87 virus. Inverse positive correlation between the virus concentration and time to detection was observed. Sample dilutions not detected were assigned arbitrary 0 (zero) values and appear on the figure as 0. A general increase in time is observed from a higher concentration ($2.9x10^8$) to a lower virus concentration ($2.9x10^1$). A sample with 2.9 copies/mL and the negative controls poly A and water were not detected (The horizontal axis is labelled from the highest virus concentration to the lowest).

4.1.2.5.3 Determination of precision for primers based on time to detection

Both intra- and inter-assay precisions were tested for all 4 sets of primers. A comparison of real time turbidimetry and visual colour detection (HND dye) methods was also performed based on time to detection. Statistical analyses were performed and results reported as recommended for medical statistics (Lang and Secic, 2006).

4.1.2.5.3.1 Intra-assay precision

The highest intra-assay precision was observed with primer sets HiLtr (mean = 38.52; CV = 1.44%) and HiGp (mean = 21.49; CV = 2.2%) when tested by turbidimetry (Figure 9). However the two exhibited lower precisions, mean = 47.33; CV = 6.5% and mean = 27; CV = 11.6% for HiLtr and HiGp respectively, when tested by the HNB dye detection method. HiInteg and HiP exhibited high precision for both detection methods. These results showed that HiInteg and HiP were potentially more stable and robust compared to HiLtr and HiGp.

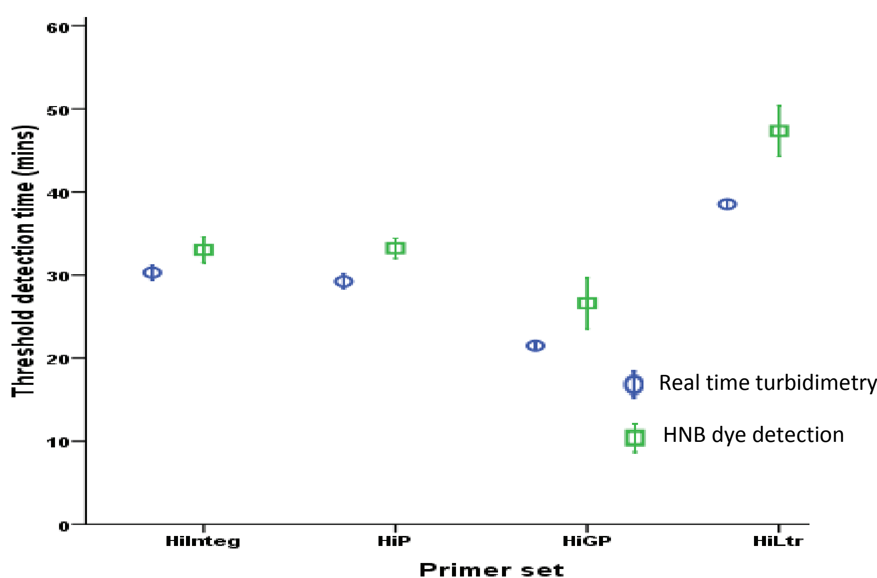


Figure 9. Results of intra-assay precision for HiInteg , HiP, HiGp and HiLtr sets of primers. The results show mean and standard deviation of time to detection for the sets of primers tested. HiLtr and HiGp exhibited the highest precisions by real time turbidimetry, but showed a reduced precision for HNB dye detection. HiInteg and HiP exhibited stability in precision for both detection methods.

4.1.2.5.3.2 Inter-assay precision

Cell culture supernatants of IIIB virus were used to test inter-assay precision and also to compare the findings based on the detection method used (real-time turbidimetry versus HNB dye detection). A higher precision was observed for real time turbidimetry when compared to the HNB detection method. Comparisons in performance between heating block and real time turbidimetry is reported in Figure 10. Within each primer set, HiGp recorded the highest precision by real time turbidimetry (mean = 21; CV = 2%) but exhibited the lowest precision by HNB dye detection (mean = 26; CV = 22%). Precisions with accepted ranges were realized for HiInteg (mean = 30; CV = 3%) and HiP (mean = 30; CV = 7%) sets of primers by real time turbidimetry and for HNB dye detection (mean = 33; CV = 9%) for HiInteg and (mean = 33; CV = 0%) for HiP sets of primers. The lowest precision was shown for HiLtr by real time detection (mean = 50; CV = 12%) and by HNB dye detection (mean = 46; CV = 17%). All CVs calculated for HiLtr were outside the variation range of 10%, which is generally established for an acceptable precision (Lang and Secic, 2006).

4.1.2.5.3.3 Inter-assay precision across subtypes

Testing was done over a period of 3 days using HIV-1 group M:B and non-B samples of known viral loads quantified by the Abbott m2000rt system, using the 4 sets of primers. The highest precision was found with HiInteg primers (Table 4) for all subtypes except for subtype F1 which, just like HiLtr primer, showed a dismal performance. Viral load estimation for this F1 subtype sample showed a low

viral load of 250 copies/mL with the Abbott m2000rt assay. Although the detection threshold for LAMP assay was not yet determined, it was highly suspected that the low viral load could have been responsible for the reduced precision as opposed to its genotype. This observation of non-genetic influence was supported by the observation that subtype F2 (which is closely related to F1 in nucleic acid sequence pattern), with a higher viral load exhibited a higher precision for all primer sets. Observations for the HiP set of primers could not be explained as this set of primers recorded high precisions with lower standard deviation for all samples including HIV-1 group O and HIV-2 samples, despite the fact that all primers were designed to target only HIV-1 group M viruses. The HiGp set detected only subtype B virus presenting the lowest precision compared to the other sets of primers.

Subtype	**Viral load**	**HiInteg**			**HiP**			**HiLtr**			**HiGp**		
HIV-1 M	**Abbott m2000rt**	***Mean time***	***SD***	***CV (%)***	***Mean time***	***SD***	***CV (%)***	***Mean time***	***SD***	***CV (%)***	***Mean time***	***SD***	***CV (%)***
A1	$6.9x10^5$	32.3	0.9	2.8	27.3	0.9	4.2	29.0	1.4	6.5	ND	-	-
B	$2.9x10^6$	30.0	0.8	3.2	26.0	1.6	7.6	28.5	1.1	4.6	41.0	3.9	9.5
D	$2.4x10^6$	32.0	0.8	2.5	29	1.7	7.3	29.0	0.3	1.1	ND	-	-
F1	$2.5x10^2$	45.1	7.1	19.3	31.0	2.9	1.2	31.0	3.4	13.7	ND	-	-
F2	$6.5x10^5$	30.0	0.7	2.4	27.0	0.7	2.7	28.5	4.7	1.8	ND	-	-
G	$1.1x10^5$	33.0	0.6	2.0	27.0	0.5	2.1	28.0	2.7	11.8	ND	-	-
HIV-1 O	$6.2x10^4$	ND	-	-	28.2	2.3	10	30.0	1.6	6.7	ND	-	-
HIV 2	$5.0x10^5$	ND	-	-	32	1.3	4.9	ND	-	-	ND	-	-

Table 4. Inter- assay precision for the 4 sets of primers among HIV-1 M:B and non B subtypes. Overall HiInteg primers exhibited higher precision (with the lowest % coefficient of variation (CV)) across all subtypes except for HIV-1 M:F1 compared to HiP and HiLtr sets of primers. The lower precision among F1 subtype by HiInteg was attributed to potentially low viral load. The HiGp set of primers only detected subtype B. Samples not detected are indicated as (ND) followed by dashes (-)

4.1.2.6 Real time turbidimetry versus HNB dye detection

An independent T-test was performed to compare the time to threshold (Tt) values obtained by turbidimetry and HNB dye detections. No significant difference in the results of turbidimetry detection (mean = 33, SD = 11) compared to HNB dye detection (mean = 35, SD = 9); $t(22) = -0.49$

was observed. It was also observed that in each case except for HiLtr, time to threshold detection was achieved earlier by turbidimetry compared to HNB dye detection (Figure 10).

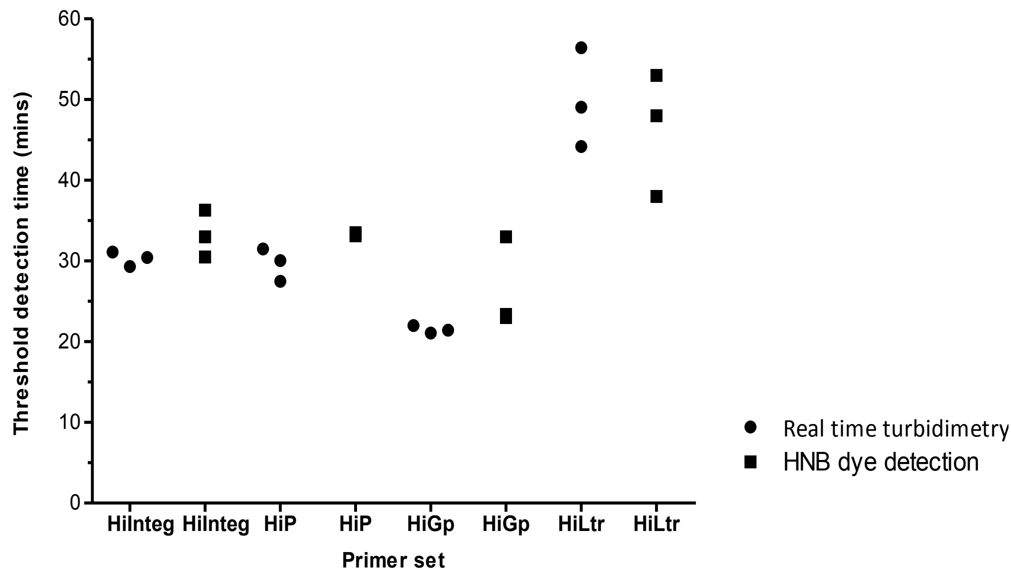


Figure 10. Comparison of 2 detection methods (real time turbidimetry and HNB dye detection) for cell culture supernatant from HIV-1 IIIB sample. An earlier detection time by real time turbidimetry was observed for all primer sets except for HiLtr compared to HNB dye detection.

4.1.2.7 Evaluation of primer mismatches

Following the discrepancies by the 4 primer sets on analytical sensitivity and inter-assay precision, testing for sequence variations within subtypes (using HXB2 as reference strain) was done by aligning primer specific targets (F3, B3, FIP, BIP, LF and LB) employing an online HIV sequence alignment tool (http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html) from the Los Alamos National Laboratory.

HiInteg primer set recorded the minimum average number of mismatches among non-B (A, C, D) subtypes, and at the same time showed the highest number (25) of mismatches with HIV-1 group O strains (Table 5). Among subtype B strains within which all the primers had been designed to target, the number of mismatches was as follows: HiInteg set = 1, HiP = 9, HiLtr and HiGp sets each = 14. The highest number of mismatches were mainly observed within the inner primers (FIP and BIP), whereas the least number of mismatches were observed within the outer (F3 and B3) and the loop (LF and LB) primers.

Primer Set	HIV-1 group M Subtype	Number of mismatches at targets						Total No. Mismatches
		F3	B3	FIP (F1c+F2)	BIP (B2+B1c)	LF	LB	
HiP	A	2	3	4	4	3	1	17
	B	1	2	1	3	1	1	9
	C	1	2	2	3	0	2	10
	D	1	2	2	5	0	1	11
	O	1	2	5	6	4	2	20
HiInteg	A	3	1	1	1	1	0	7
	B	1	0	0	0	0	0	1
	C	3	2	0	1	1	0	7
	D	1	1	0	0	0	0	2
	O	7	3	1	6	3	5	25
HiLtr	A	1	3	5	4	0	3	16
	B	1	3	3	4	1	2	14
	C	3	4	4	6	1	2	20
	D	2	5	4	5	0	3	19
	O	3	5	7	6	0	3	24
HiGp	A	5	3	4	7	5	3	27
	B	4	1	2	3	3	1	14
	C	4	3	4	7	4	3	25
	D	4	1	3	2	3	1	14
	O	1	2	5	8	4	3	23

Table 5. Mismatches within primer targets for the 4 LAMP primer sets. Results show the average number of mismatches for the target sequences of different primer sets when aligned by an online alignment tool obtained from the HIV sequence database from Los Alamos National Library (http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html). The least average number of sequence mismatches was shown by HiInteg set, whereas the highest numbers were shown by HiLtr and HiGP primer sets. Among HIV-1 M subtype B (in blue dotted boxes) HiInteg recorded a single mismatch, whereas HiLtr and HiGp recorded the highest numbers of mismatches of 14. Sequence alignments were based on a reference strain (HXB2) of HIV-1 group M subtype B.

4.1.2.8 Selection of primers for further evaluation

Based on the results of analytical sensitivity and precision, HiInteg and HiP targeting the integrase and the p24 genes respectively were selected for further evaluations and comparisons in the LAMP assay. The aim was to select the most appropriate primer set which could later be used for quantification of clinical samples by the LAMP assay.

4.1.2.9 Evaluation of the sample condition

4.1.2.9.1 Testing the matrix effect of plasma and aqueous solution

Plasma samples were spiked with virus from cell culture supernatants and analyzed by real time turbidimetry. The means of values for spiked plasma and diluted cell culture supernatants (of identical virus concentrations) were compared in order to test whether the type of matrix tested influenced the rate of detection by LAMP assay. The rationale for testing matrix was due to the fact that under limited logistical conditions, it is sometimes not possible to obtain the required amount of plasma for analysis. In such a scenario it would therefore be prudent to dilute the samples in negative plasma or diluent in order to obtain the required volume for analysis. The findings on the matrix effect would therefore be very instrumental in establishing LAMP under limited logistical conditions.

An independent T-test was performed to compare the time to threshold (Tt) values for plasma spiked samples and RPMI 1640 culture medium samples. There was no significant difference for plasma spiked samples (mean = 27, SD = 14) and RPMI 1640 medium samples (mean = 28, SD = 12); $t(48) = 0.09$. The finding thus indicated that there was no significant influence of the matrix when negative plasmas were spiked with viruses from cell culture supernatants. Independent testing of the samples by the different primer sets also showed no significant difference between HiP primers (mean = 29, SD = 7) and HiInteg primers (mean = 26, SD = 17); $t(48) = 0.9$. Results therefore confirmed that regardless of the primer set used no significant influence of the matrix could be observed for the results generated by the LAMP assay.

4.1.2.9.2 Specificity for HiInteg and HiP primers with negative clinical samples

HiInteg and HiP primers were tested with HIV negative fresh plasma samples obtained from in-house volunteers. 70 µL of whole blood from these volunteers was also used to process DBS before they were tested together with their corresponding plasmas. The 5 HIV negative plasmas and their corresponding DBS were tested 7 times resulting in a total of 35 tests for each category. 3 HIV positive plasmas together with their corresponding DBS were tested 5 times resulting in a total of 15 tests in each category. The positive controls were samples with low viral loads selected with the aim of minimizing the probability of cross contamination. HiInteg primers exhibited 100% specificity (Table 6) for each category. HiP exhibited reduced specificities ≥89% for plasma and ≥91% for DBS due to false positive detections (Table 7). Although not being the main focus, the lower sensitivity realized for HiInteg primers was expected since previous findings on analytical sensitivity and precision analysis had shown reduced sensitivity for lower viral loads.

Test parameter	Primer set			
	HiInteg		*HiP*	
	Plasma	*DBS*	*Plasma*	*DBS*
Sensitivity % (CI): **n =15**	40% (16 - 68)	7% (1 - 32)	60% (32 - 84)	67% (38 - 88)
Specificity % (CI) : **n =35**	100% (90 - 100)	100% (90 - 100)	89% (73 - 97)	91% (77 - 98)
PPV %	100% (54 - 100)	100% (17 - 100)	69% (39 - 91)	77% (46 - 95)
NPV %	80% (65 - 90)	71% (57 - 83)	84% (68 - 94)	86% (71 - 95)

Table 6. Specificity analysis of HiInteg and HiP sets of primers for selected clinical samples. HiP set showed a reduced specificity and predictive values as a result of false positive detections. The low sensitivity by HiInteg set was expected since the 3 positive clinical samples with low viral loads were selected to reduce the risk of cross contamination. Specificity results were calculated within 95% confidence interval (CI)

HiP set of primers showed earlier positive results compared to HiInteg set, for all detected samples (Table 7), and in 2 cases HiP set detected even samples <1,000 copies/mL. Although previously false positive results had been observed due to contamination, contamination was ruled out for these results shown by HiP, since not all negative samples including negative controls were falsely detected by the set. False negative detections were also random within the 3-day period of analysis. Furthermore positive controls of low viral loads had been carefully selected to minimize the risks of contamination. The finding of a potential low specificity with HiP primers provided a preference for HiInteg primers for the detailed analysis of clinical samples.

Sample ID	Patient details		Plasma		Dry Blood Spot	
	VL (Abbott)	Sex	Time to detection(mins)		Time to detection (mins)	
	Copies/mL		*HiInteg*	*HiP*	*HiInteg*	*HiP*
N001C	0	Male	>60	>60	>60	39
N002C	0	Male	>60	37.06	>60	39
N003C	0	Male	>60	37.24	>60	33.54
N004C	0	Female	>60	43.06	>60	34.24
N005C	0	Female	>60	>60	>60	>60
W001C	0	Female	>60	>60	>60	>60
P001C	2418		48.18	30.18	50.3	26.12
P002C	753		>60	>60	>60	36.48
P003C	612		>60	31	>60	>60

Table 7. False positive results obtained by the HiP primer set for HIV negative plasma and DBS. False positive results (in square red boxes) by HiP were realized for 4 samples, while 1 positive sample was not detected in each sample category. False positive results occurred only with Tt values >30 minutes. In the previous analysis HiP showed high sensitivity with a detection time ≤30 minutes for positive samples. These results prove that weak positive results by HiP primers with Tt values >30 minutes should be interpreted with caution. Tt – time to threshold detection, Tt >60 meant no signal was obtained within 60 minutes. Viral load (VL) was quantified by Abbott m2000rt system.

4.1.3 Evaluation of HiInteg primers for potential use in analysis of clinical samples

After pre-analysis of the HiInteg set of primers was selected for eventual use in semi-quantitative detection of HIV-1 M:B and non-B subtypes, due to the qualities it showed compared to the other 3 sets of primers.

4.1.3.1 Plasma, dry blood spots and dry plasma spots

Studies have reported potential usability of LAMP technology with non-extracted plasma and whole blood samples (Curtis et al., 2008; Curtis et al., 2009). A total of 18 positive plasma samples, 2 negative samples and their corresponding dried blood spot samples were selected for testing. These samples were then categorized as (i) nucleic acid extracted plasma samples (ii) heated non-extracted plasma samples (iii) non-extracted plasma samples treated with a lysis buffer and (iv) dry blood spot samples, based on their mode of treatment before analysis with LAMP assay.

i. **Nucleic acid extracted plasmas:** Samples were treated as described in chapter 3.2.2.2.1.1 under materials and methods and then used for comparisons during evaluations in steps iii and iv.

ii. **Nucleic acid extracted dry DBS:** Samples were treated as described in chapter 3.2.2.2.1.2 under materials and methods and used for comparisons during evaluations in steps iii and iv.

iii. **Heated non-extracted plasma samples:** A total of 8 HIV positive plasmas (which included 5 samples detected by the Abbott m2000rt system and 3 samples below detection threshold) and 2 negative plasmas were heat-treated and tested by real time turbidimetry together with their corresponding extracted plasma and dry blood spot. All (5/5) the samples above detection threshold, none (0/3) below detectable threshold and none (0/2) of the negative samples were detected by LAMP among extracted plasmas. Similarly all (5/5) the samples above detection threshold, none (0/3) below detectable threshold and none (0/2) of the negative DBS samples were detected among the extracted DBS. In contrast none (0/8) of the heat-treated non-extracted plasmas using either 5.5 µL or 9.5 µL could be detected by LAMP assay.

iv. **non-extracted plasma samples treated with a lysis buffer**: A total of 9 HIV positive samples and their corresponding extracted plasma and DBS were analyzed. In total, 6/8 extracted plasmas, 5/8 DBS and none (0/8) of the non-extracted plasmas were detected by LAMP assay.

Although LAMP assay showed the potential for use by both extracted plasma and dry blood spot samples, it did not display this ability with non-extracted plasma samples.

4.1.3.2 Nucleic acid sequence analysis of primer target regions

Using different sequences generated in-house and from the HIV database, sequences of various HIV-1 group M:B and non-B, HIV-1 group O and HIV-2 were selected and aligned with the primer binding sites to check for anticipated primer mismatches. Sequences for HIV-1 group O and HIV-2 were selected purposely for comparisons and due to the fact that these samples had not been detected by HiInteg primers. The results show an average of 3 mismatches for HIV-1 M:B and non-B subtypes at the outer forward (F3) region, 1 mismatch on outer reverse (B3) and an average of 1 mismatch for the inner primers (FIP and BIP) across all the subtypes (Figure 11a and Figure 11b). Loop target primers show an average of 1 mismatch. Contrary to findings for HIV-1 group M, group O (Figure 11c) and HIV-2 (Figure 11d) show in average ≥22 mismatches indicating that this set of primers is not suitable for detecting the RNA of these viruses (see also page 61).

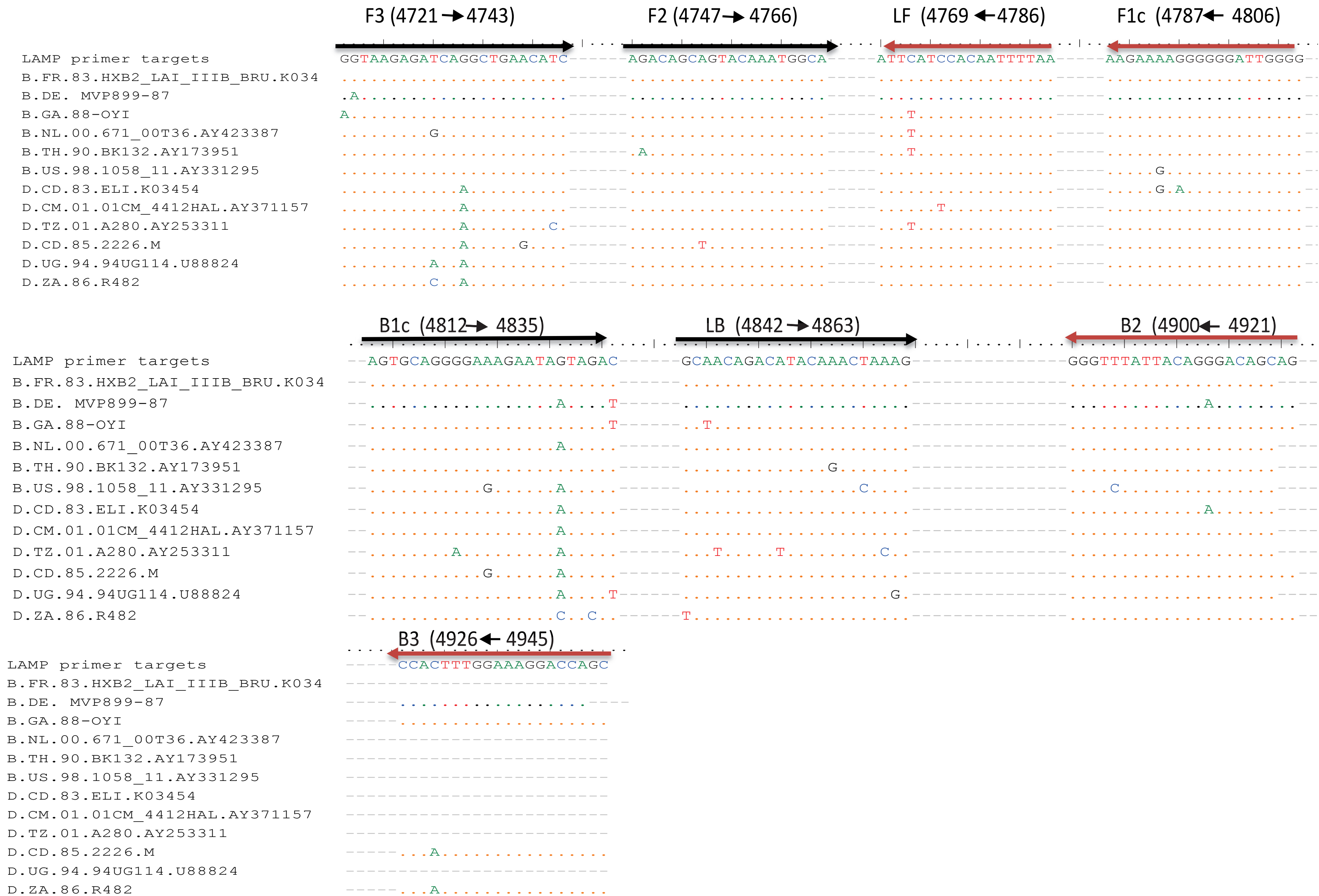


Figure 11a. Nucleic acid sequence alignment patterns of HIV-1 M subtypes B and D viruses. HiInteg primers show an average of 1 mismatch per subtype within B and D subtypes. Molecular characterization has shown subtypes B and D to be sub-subtypes (Los Alamos, 2008). Epidemiologically subtype D is found in Kenya. This primer set was designed according to the IIIB virus sequence. Incomplete sequences are shown by dashes (non dots) at the primer targets.

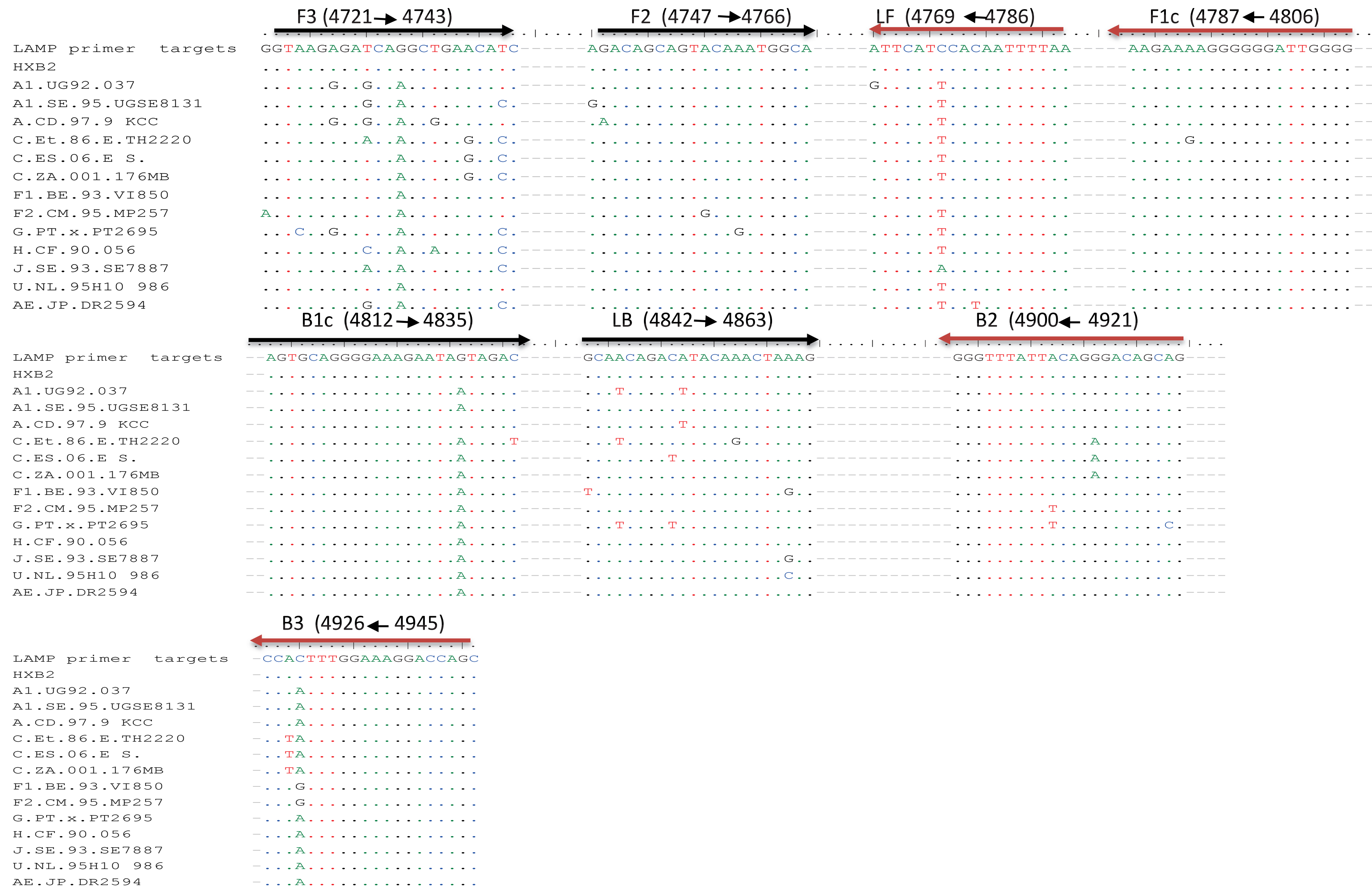


Figure 11b. Nucleic acid sequence alignment of selected HIV-1 M: non B, non D isolates with HiInteg LAMP primers. An average of 3 mismatches was detected for the F3 forward primer. F3: Forward primer, F2: Forward inner primer, LF: Loop forward, F1c: Forward inner reverse complementary primer, B1c: Backward inner reverse complementary primer, LB: Loop backward, B2: Backward inner primer, B3: Backward primer. FIP (F1c+ F2), BIP (B2 + B1c). Subtype A and C are prevalent in Kenya.

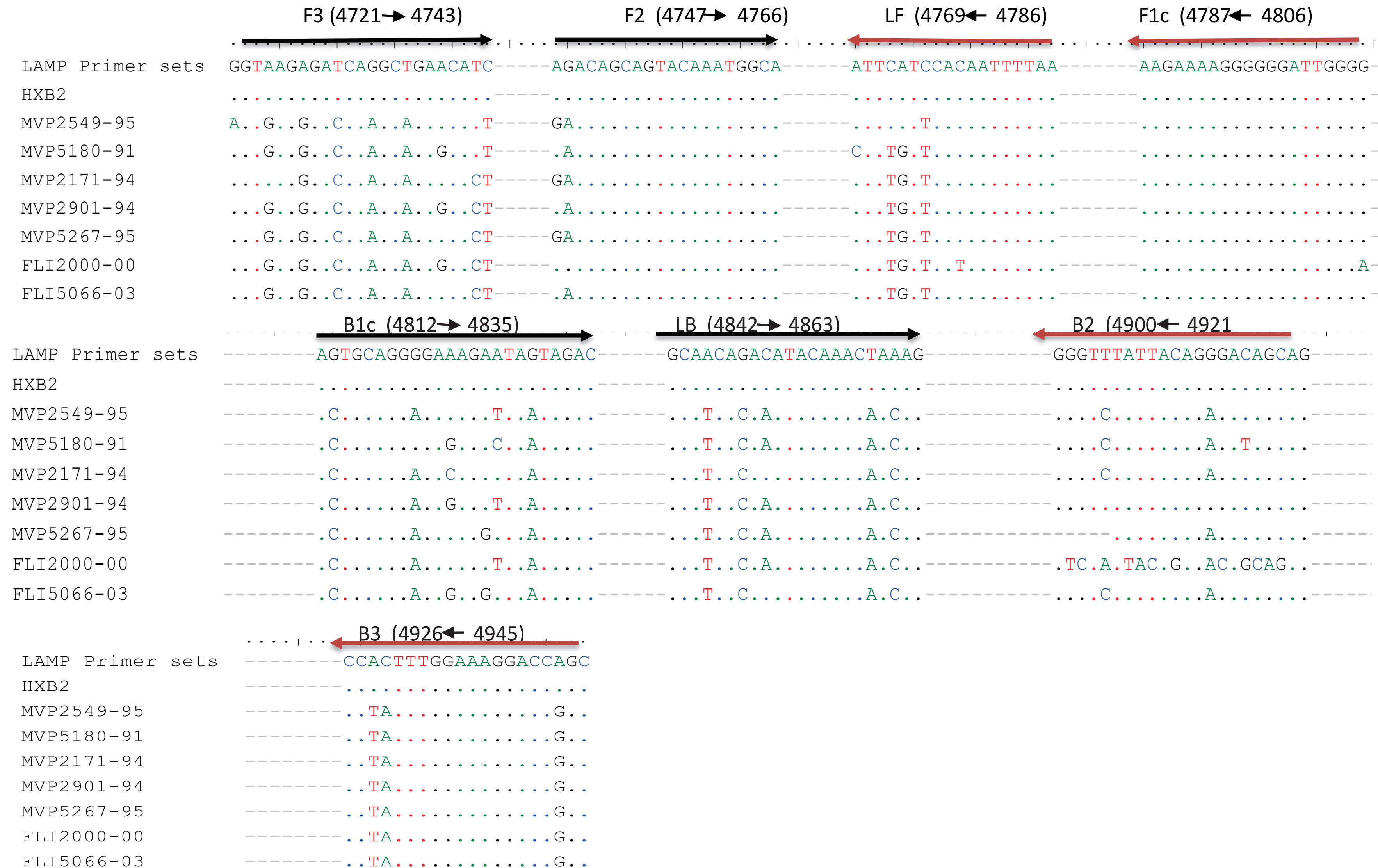


Figure 11c. Nucleic acid sequence alignment of HIV-1 group O sequences with HiInteg primers. The results show mismatches at the outer primers F3 and B3 and the inner primers (FIP and BIP). Mismatches at these 2 positions were considered as the reasons for insufficient binding of HiInteg primers among group O viruses. F3: Forward primer, F2: Forward inner primer, LF: Loop forward, F1c: Forward inner reverse complementary primer, B1c: Backward inner reverse complementary primer, LB: Loop backward, B2: Backward inner primer, B3: Backward primer. FIP (F1c + F2), BIP (B2 + B1c). Until now HIV-1 group O has not been detected in Kenya.

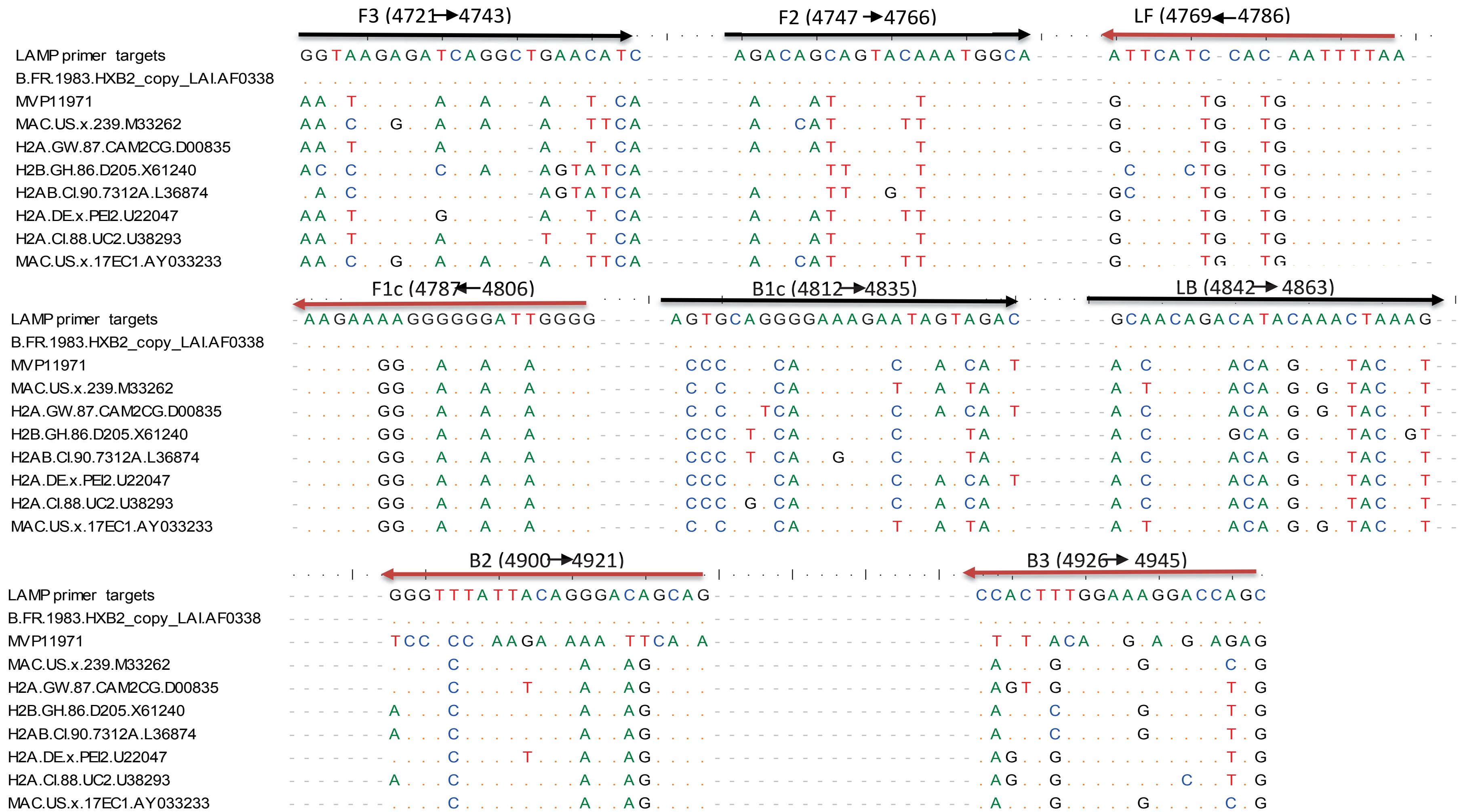


Figure 11d. Nucleic acid sequence alignment of HIV-2 with HiInteg set of primers. Results show an average mismatch of ≥5 per primer target. The high number of mismatches shows insufficient primer binding and thus the failure to detect HIV-2 virus RNA. F3: Forward primer, F2: Forward inner primer, LF: Loop forward, F1c: Forward inner reverse complement primer, B1c: Backward inner reverse complement primer, LB: Loop backward, B2: Backward inner primer, B3: Backward primer. FIP (F1c + F2), BIP (B2 + B1c). HIV-2 is not prevalent in Kenya

4.2 Analytical sensitivity and estimation of lower threshold limits

4.2.1 Analytical sensitivity

Analytical sensitivity (ASe) refers to the minimum number of copies in a sample that can be measured accurately with an assay. The standard performance parameters of diagnostic quantitative assays are the lower limit of detection (LLOD) and the lower limit of quantification (LLOQ). The LLOD is the lowest amount of the analyte detectable by an assay in a single reaction with a certain degree of certainty, whereas the LLOQ refers to the lowest amount of the analyte which can be reproducibly quantified. Methods for determining LLOD and LLOQ in a chemical reaction have previously been defined (Haeckel et al., 1998; Mikkelsen and Cortón, 2004), however they have been found unsuitable for quantitative assays. Guidelines from the World Organization of Animal Health recommend that LLOD be determined as the end point dilution at which 50% of the tested samples are positive (World Organization for Animal Health (OIE), 2013).

Using an in-house software (Ceres) developed by the Virus Diagnostic Department of the Max von Pettenkofer-Institute, half maximal effective concentration (EC 50) of viral load panels of ($0.2x10^1$ to $2.9x10^6$ copies/mL) of IIIB and MVP899-87 viruses (tested by Abbott m2000rt system) was determined in the LAMP assay. The EC 50 was used as an estimator for LLOD, whereas the LLOQ was estimated at a viral load in which ≥95% detection by LAMP was achieved. Samples were tested between 10 to 18 times by the LAMP assay and the percentage of positive detections calculated.

The LLOD was attained at $1.2x10^3$ copies/mL (Figure 12) whereas LLOQ was attained at a concentration of $9.8x10^3$ copies/mL. It was also observed that viral loads of above $3x10^3$ copies/mL were detected at the rate of more than 70%. These results are consistent with those already obtained earlier where samples with low viral loads presented a reduced precision, with only a few detected or detection obtained after a repeat test.

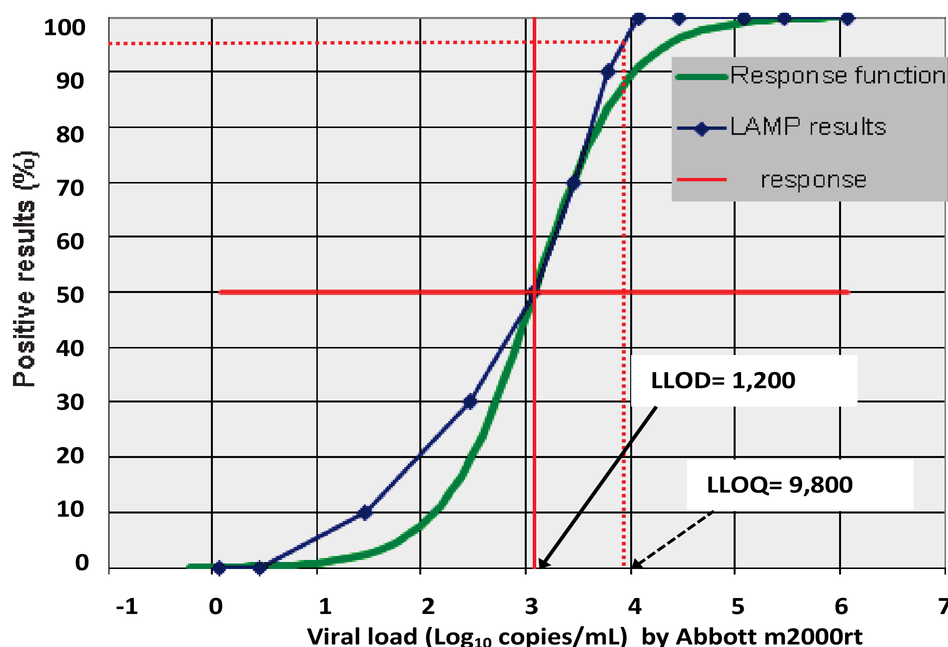


Figure 12. Estimation curve for lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for LAMP compared to the Abbott m2000rt system. LAMP response rate (vertical axis) was tested using HiInteg set of primers for cell culture supernatants of IIIB and MVP899-87 representing HIV-1 M subtype B virus. The figure shows half maximal concentration (EC 50) for detection, which estimates LLOD by LAMP, detected at 1,200 copies/mL. The lower limit of quantification (LLOQ) estimated at 95% detection rate (red dotted lines) was reached at 9,800 copies/mL.

4.2.2 Generation of a mathematical model for estimation of viral load by LAMP assay

A major feature used in the evaluation of the results by LAMP technology is the generation of large amounts of magnesium pyrophosphate deposits as a visible white precipitate (Mori et al., 2001), which is produced in proportion to the amount of amplified products. Real time turbidimetry of the by-product can be measured and the results estimated based on the time to threshold (Tt) detection.

Since linearity of the assay based on time had been realized across a dilution panel from HIV cell culture supernatant of IIIB sample, the next part of this study was then to test for a linear relationship between the known viral load and time to detection. Viral loads for IIIB and MVP899-87 were first quantified by the Abbott m2000rt system and 10-fold dilutions for each sample material made from higher dilutions of 2.9×10^6 and 1.2×10^6 copies/mL. Virus concentrations therefore ranged from 0.12×10^1 to 2.9×10^6 copies/mL. Testing was done 4 times by LAMP turbidimetry and the results obtained were used to test for correlation between time and viral load and eventually to generate a regression equation for estimation of corresponding viral loads in LAMP. Calculations for estimation parameters were done using MS-Excel version 14 (a computer package from Microsoft) http://www.microsoft.com/en-us/download/details.aspx?id =5829.

Since the task was to compare and relate an assay for semi-quantitative detection to a purely quantitative, two estimation curves, a linear curve y = -2.263 ln(x) + 59.309 and a non-linear curve y = $63.526x^{-0.067}$ (Figure 13) were generated and used for viral load estimations. From the results it was realized that the average time for $1\log_{10}$ difference was 3 minutes. The 3 minute interval however was found to increase as the viral load decreased.

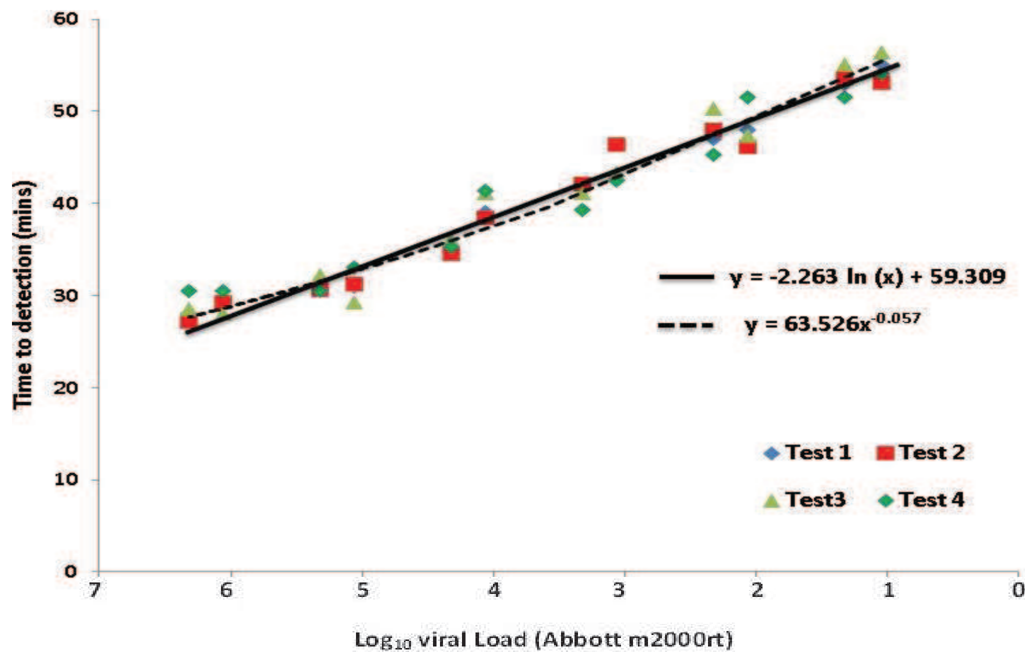


Figure 13. Estimation curves for viral load quantification by LAMP assay. Results show a linear estimation curve (straight line) and a non-linear estimation curve (dotted line) both generated by HiInteg primer sets using HIV cell culture supernatant of IIIB virus. A high regression coefficient of 0.99 was generated for both curves indicating a negligible dispersion from the regression line between viral load measured by Abbott m2000rt system and time to detection by LAMP assay. An average of 3 minutes was found as the difference between a $1\log_{10}$ difference. Testing was done within a span of 4 days. Tests 1, 2, 3 and 4 represent the results generated for each day.

No significant difference was found for the viral loads estimated from time by the two curves when 20 different samples with known viral loads were tested by LAMP and the estimated viral loads compared with those of the Abbott m2000rt system. In each case a correlation R_s (20) = 0.8 and a linear regression R^2 (20) = 0.99 were achieved between Abbott m2000rt and the LAMP assay. These results therefore showed that regardless of the estimation model used between the two estimation curves no major difference would be realized, and if any, it would be insignificant to alter the results by a wider margin of estimation. The linear estimation curve was thus preferred for subsequent estimations, mainly due to its ease of use.

4.2.3 Quality control

A quality control test was performed to check the linearity of the LAMP viral load values in comparison to the viral loads determined by the Abbott m2000rt system using the linear estimation curve. Cell culture supernatants of IIIB and MVP899-87 samples with viral loads of approximately 10 to 2.1×10^7 copies/mL were used. The results obtained showed again a linear correlation (as had previously been shown in Figure 13) between the two groups of LAMP viral loads and the viral loads of the Abbott m2000rt system (R^2 (11) = 0.99 for IIIB and R^2 (11) = 0.98 for MVP899-87) as shown in Figure 14. The results further confirmed the reliability of the LAMP assay for samples with high viral loads which also showed high concordance between the two assays.

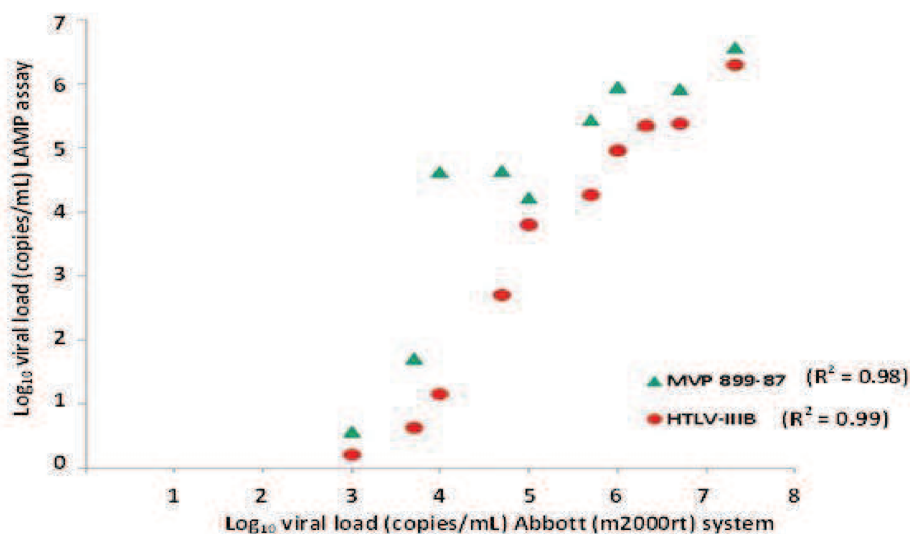


Figure 14. Quality control tests for assessment of linearity. Results show a linear correlation between LAMP and Abbott m2000rt assay for cell culture supernatants of IIIB, R^2 (11)= 0.99 and MVP899-87, R^2 (11) = 0.98 viruses.

4.2.3.1 Precision tests based on estimated viral loads generated by linear estimation curve

4.2.3.1.1 Intra- and inter-assay precision testing based on viral load

A total of 58 tests of cell culture supernatants of varying viral load measurements by Abbott m2000rt were tested in different frequencies during the same runs by LAMP assay, and the viral loads obtained used to evaluate the intra-assay precision. Similarly, 21 tests of cell culture supernatants of varying viral loads were tested on 3 different days and the LAMP viral loads used to evaluate inter-assay precision. All the viral load values obtained by LAMP were first transformed into a log scale for ease of precision analysis. The precision was evaluated based on standard deviations and not based on coefficient of variations as recommended for viral load test assays (Bustin et al., 2009).

Apart from 12 samples within the viral load range of $3\log_{10}$ and 9 samples within the range of $4\log_{10}$ which presented $1.3\log_{10}$ and $1.2\log_{10}$ deviations for intra-assay and inter-assay respectively, all other viral load samples were within the accepted $1\log_{10}$ standard deviation range. In both cases the lowest standard deviations were recorded within viral loads of $5\log_{10}$ copies/mL indicating a strong correlation between LAMP and Abbott m2000rt system within this margin. The results realized for samples with $3\log_{10}$ viral load values were in concordance with those realized during the precision analysis where lower viral loads presented lower precision in detection. Further the LLOQ for this assay was calculated at 9.8×10^3 copies/mL whereas the LLOD was calculated at 1.2×10^3 copies/mL, results which could also explain the log difference realized for this group of samples, since detection of a sample would not necessarily mean precise quantification. As shown in Table 8 and Table 9 there was a general decrease in the variations (hence increased correlation) between Abbott m2000rt system and LAMP assay as the viral load increased, suggesting a higher precision and increased rate of viral detection at higher viral loads.

Test Statistic	**Results**				
	Viral load $\log_{10}$(cop/mL)	**Frequency of testing**	**Mean $x\log_{10}$**	**standard deviation x $\log_{10}$**	**Coefficient of variation (%)**
Intra-assay precision	1	N/A	-	-	-
(n = 58)	2	4	2.1	0.5	23
	3	12	2.9	1.3	46
	4	14	4	1	24
	5	16	5.4	0.5	9
	6	12	4.73	0.7	15
Inter-assay precision					
(n = 21)	1	N/A	-	-	-
	2	N/A	-	-	-
	3	3	1.12	1	87
	4	9	3.2	1.2	37
	5	6	5	0.4	9
	6	3	5.4	0.4	7

Table 8. Inter- and intra-assay precision test results showing log standard deviations between Abbott m2000rt system and LAMP assay. Results showed an increase in precision (shown by lower standard deviations) with higher viral loads. N/A refers to sample viral loads which were not tested, mainly due to viral loads below the threshold of detection established for LAMP assay.

4.2.3.1.2 Inter-investigator variation

A panel of HIV cell culture supernatants of various viral loads was analyzed by 2 independent investigators within a duration span of 7 days. Results were analyzed independently by the two

investigators and tested for precision and correlation. Samples with high viral loads recorded higher precisions and lower standard deviations (Table 9). A strong correlation R^2 (11) = 0.75 was also calculated for results of both investigators. Lower precisions with low viral loads as observed for the $3log_{10}$ group of samples have already been discussed. The findings show that the accuracy of this assay is investigator independent. The results however cannot be interpolated to different environmental conditions as the laboratory conditions in which the tests were done were identical for both investigators.

Test Statistic	**Results**				
	Viral load log_{10}(cop/mL)	**Frequency**	**Mean $xlog_{10}$**	**standard deviation x log_{10}**	**Coefficient of variation (%)**
Inter-investigator Precision	1	N/A	-	-	-
	2	N/A	-	-	-
	3	6	1.5	1.7	110
	4	6	3.3	0.4	11.6
	5	6	4.6	0.3	6.8
	6	6	5.0	0.2	4.8

Table 9. Inter-investigator precision between two independent investigators in the same laboratory. The results showed a higher precision (lower standard deviation) between the investigators for samples with high viral loads, and a reduced precision (standard deviation $1.7log_{10}$) for samples in the $3log_{10}$ viral load category. The findings were consistent with previous findings which have shown that the precision increases with higher viral loads.

4.2.4 Analysis of HIV-1 subtypes from cell culture supernatants

A total of 166 HIV-1 containing cell culture supernatants were tested by LAMP and Abbott m2000rt system and the results of their viral loads compared. The cell culture supernatants included subtypes A1 (n = 2), B (n = 144), C (n = 5), D (n = 5), E (n = 2), F1 (n = 2), F2 (n = 3) and G (n = 5). A positive linear correlation R_s (166) = 0.7 was obtained with a slope R^2 (166) = 0.6 for the two assay platforms (Figure 14). Generally 60% (R^2 (166) = 0.6) of the results by LAMP assay were within $\leq 1log_{10}$ of those results obtained by Abbott m2000rt system. The results also showed that viral load values obtained by the LAMP assay were lower compared to those obtained by the Abbott m2000rt system. The results also showed that $>1log_{10}$ difference between the two assays was realized mainly by HIV-1 non-B subtypes. As part of the calibration process for the LAMP assay, a standard cell culture supernatant sample of MVP899-87 virus with a viral load of $2.1x10^6$ copies/mL was used as an internal positive control. The expected time to threshold detection of this internal control by LAMP was between 28.30 to 30.0 minutes.

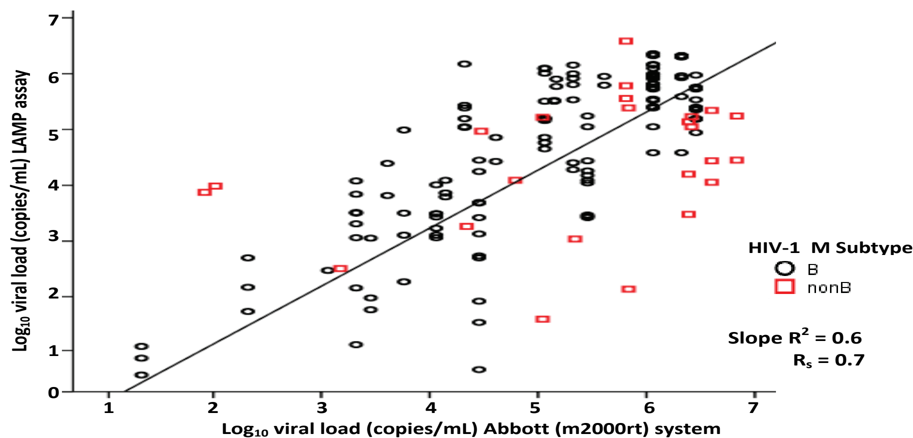


Figure 15. Correlation analysis for cell culture supernatant samples of various HIV-1 subtypes. A direct linear correlation R_s= 0.7 between LAMP assay and Abbott m2000rt was observed. The results showed 60% of the viral load measurements within the regression slope (±log_{10} copies/mL). These results also indicated potential challenges with subtype specificity for LAMP assay with HiInteg primers.

4.3 Testing and analysis of clinical patient samples with LAMP assay

Clinical samples tested in this study were categorized into four main categories namely,

i. Kenyan plasma samples analyzed in Nairobi
ii. Kenyan plasma samples analyzed in Munich
iii. Kenyan Dry Blood Spot samples analyzed in Munich
iv. Munich plasma samples analyzed in Munich.

i. Kenyan plasma samples analyzed in Nairobi: This panel consisted of 135 samples (87 HIV positive and 48 HIV negative) with viral loads between 0 (undetectable) to 10^6 copies/mL tested by Abbott m2000rt system. HIV negative samples were first tested by rapid diagnostic kits (Determine ®) routinely used in Kenya for HIV 1/2 antibody detection. Anti-HIV negative plasmas and those undetected by Abbott m2000rt system were used to test the specificity of the LAMP assay.

ii. Kenyan plasma samples analyzed in Munich: This panel included 99 HIV-RNA positive plasmas obtained in Nairobi and shipped to Munich within a period of 6 months after collection. In Nairobi, the samples were stored at -80°C prior to shipment. Upon arrival in Munich, the samples were stored at -20°C and tested within 3 months. Prior to testing by LAMP assay, the viral loads of these samples were retested in Munich by Abbott m2000rt system which was used as “gold standard” for evaluating LAMP assay. 23 samples from this group had undetectable viral load when retested in Munich despite having been positive in

Nairobi, whereas 77 samples were detected by the Abbott m2000rt system. The 23 undetected samples were used to test specificity of the LAMP assay within this category.

iii. **Kenyan Dry Blood Spot samples analyzed in Munich:** The panel included 121 dry blood spot (DBS) samples which had previously been processed in Kenya and stored at -20°C before shipment to Munich. Extracts from these samples were first tested by Abbott m2000rt system and afterwards analyzed using the LAMP assay.

iv. **Munich plasma samples analyzed in Munich**: This was the last panel and included 112 samples (92 HIV positive and 20 negative samples) obtained from the local population. 27 samples of the 92 HIV positive plasmas were dilutions of HIV positive plasma in negative plasma. The negative samples were used to estimate the overall specificity of the LAMP assay within this category of samples.

4.3.1 Sensitivity and specificity of LAMP assay with clinical samples

4.3.1.1 Overall sensitivity and specificity of LAMP assay within sample categories

Values generated by LAMP assay were used to calculate sensitivity, specificity and predictive values for LAMP assay. Sensitivity values realized ranged between 82 and 93% (Table 10) for plasma samples while a lower sensitivity of 76% was realized for DBS. A high rate of false positive results was also obtained with DBS reducing the specificity of LAMP to 77% within this category. In general, a higher specificity (99-100%) was realized for plasma samples compared to DBS.

Sample category	N	Tests			
		Sensitivity (%)	***Specificity (%)***	***PPV***	***NPV***
Kenya in Nairobi					
(plasma)	n =135	82 (51/62)	99 (72/73)	98	87
CI		*70 - 91*	*93 - 100*	*90 - 100*	*78 - 93*
Kenya in Munich					
(plasma)	n =99	86 (66/77)	100 (22/22)	100	67
CI		*76 – 93*	*84 – 100*	*95 - 100*	*48 – 82*
DBS	n =121	76 (45/59)	77 (48/62)	76	77
CI		*63 - 86*	*65 - 87*	*63 - 86*	*65 – 87*
Munich in Munich					
(plasma)	n = 112	93 (86/92)	100 (20/20)	100	77
CI		86 - 98	83 – 100	96 - 100	56 - 91

Table 10. Sensitivity, specificity and predictive values of LAMP for plasma and dry blood spot (DBS) samples. Results show a high sensitivity (82 and 93%) for plasma samples and a reduced sensitivity and specificity (76% and 77% respectively) for DBS samples. The low specificity for DBS samples was attributed to a high number of false positive results. The evaluations were carried out at 95% confidence interval (CI). **PPV –** positive predictive value; **NPV –** Negative predictive value; **n –** number of samples.

4.3.1.1.1 Sensitivity and specificity for samples of viral loads above 5,000 copies/mL

Success for HIV treatment in limited resource settings is indicated by viral loads ≤1,000 copies/mL, while treatment failure is considered for viral loads ≥5,000 copies/mL within 3 consecutive tests after start of treatment (World Health Organization, 2010a). Results of this study also showed >70% detection rate of LAMP for viral loads ≥3,000 copies/mL. Sensitivity analysis was performed focusing on samples whose viral loads would indicate treatment failure. This would be important mainly to target this group of patients potentially failing treatment. Calculation of the modified results showed an improved sensitivity of 95-100% for plasma samples (Table 11). There was also a slight improvement in sensitivity for DBS samples.

Sample category	**N**	Calculated at 95% CI		
		Sensitivity (%)	***PPV***	***NPV***
Kenya in Nairobi				
(plasma)	n =41	100 (41/41)	98	100
CI		*91 - 100*	*90 - 100*	*95 - 100*
Kenya in Munich				
(plasma)	n =43	95 (41/43)	100	92
CI		*84 – 99*	*95 - 100*	*73 – 99*
DBS	n =20	80 (16/20)	84	76
CI		*55 - 83*	*68 - 94*	*50 – 93*
Munich in Munich				
(plasma)	n = 61	98 (60/61)	100	96
CI		91 - 100	96 - 100	78 - 99

Table 11. Sensitivity for samples with viral loads ≥ 5000 copies/mL. Results show an increase in sensitivity mainly for all plasma samples (red rectangular box). CI- 95% confidence interval, PPV – positive predictive value, NPV –Negative predictive value, n – number of samples.

4.3.2 Analysis of correlation between Abbott m2000rt and LAMP assay

Results generated for the different categories of clinical samples by the LAMP assay were further subjected to correlation analysis to compare the two quantification assay parameters. The following correlation results were obtained; Kenyan plasma samples analyzed in Nairobi R_s (62) = 0.9 (Figure 16); Kenyan plasma samples analyzed in Munich R_s (77) = 0.84 (Figure 17); Kenyan DBS samples analyzed in Munich R_s (59) = 0.2 (Figure 18); Munich samples analyzed in Munich R_s (92) = 0.8 (Figure 19). One Kenyan sample (NYK73163) analyzed in Munich (Figure 17) could not repeatedly be quantified despite showing viral loads of $1x10^6$; while one Munich sample (V1221944) of viral load of $1x10^5$ copies/mL (Figure 19) was sub-optimally quantified by the LAMP assay. The 2 samples were sequenced at the integrase (IN) region to determine their subtypes and evaluate their

sequence patterns for any mutations in order to yield parameters for explanation of reduction in sensitivity and a possible improvement of the LAMP assay.

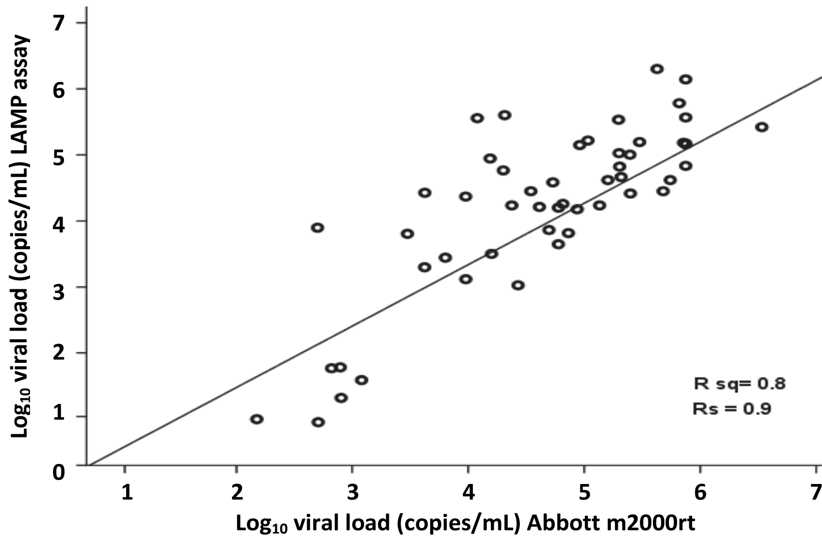


Figure 16. Correlation analysis for **Kenyan plasma samples analyzed in Nairobi**. Results showed a strong linear correlation (R_s (62) = 0.9) between the viral loads obtained by Abbott m2000rt system and LAMP assay. Similarly a regression analysis resulted in R^2 = 0.8 showing that ≥80% of the LAMP assay results fitted within the results obtained by the Abbott m2000rt system.

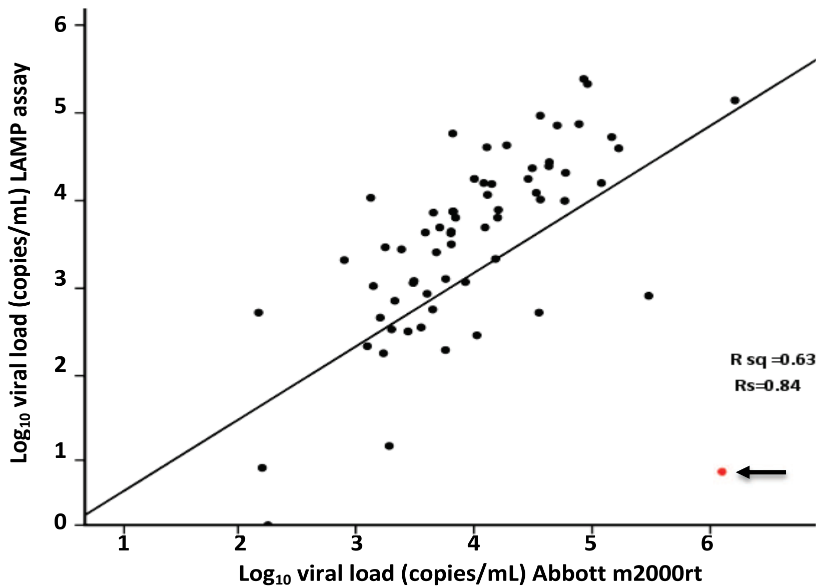


Figure 17. Correlation analysis for **Kenyan plasma samples analyzed in Munich**. Results showed a direct linear correlation (R_s (77) = 0.84) between the viral loads obtained by Abbott m2000rt system and LAMP assays. A regression analysis resulted in R^2 = 0.63 showing that ≥63% of the LAMP assay results fitted within the results obtained by the Abbott m2000rt system. Sample NYK73163 (shown in red; pointed by an arrow) could not effectively be quantified by LAMP despite a viral load ≥1x10^6 copies/mL by Abbott m2000rt system.

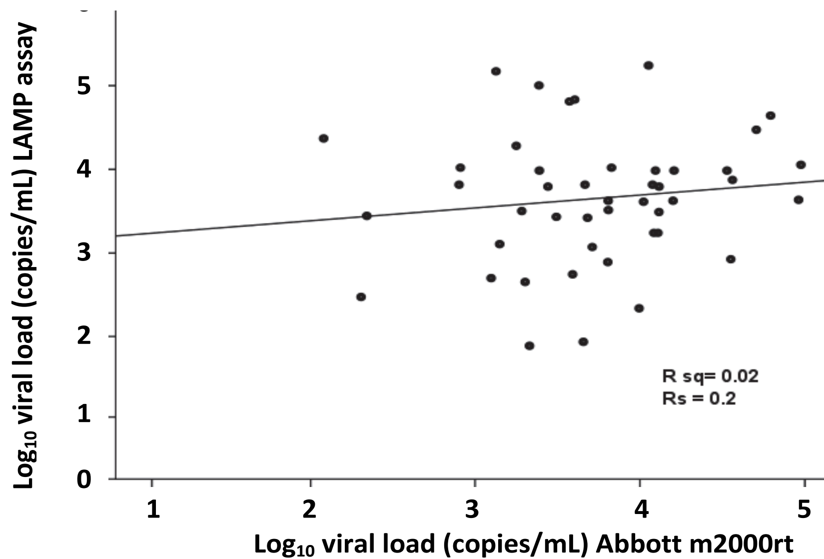


Figure 18. Correlation analysis for **Kenyan dry blood spot (DBS) samples analyzed in Munich.** Results showed no correlation (R_s (59) = 0.2) between the viral loads obtained by Abbott m2000rt and LAMP assays. Similarly a regression analysis resulted only in R^2 = 0.02 showing that only 2% of the LAMP assay results fitted within the results obtained by the Abbott m2000rt system.

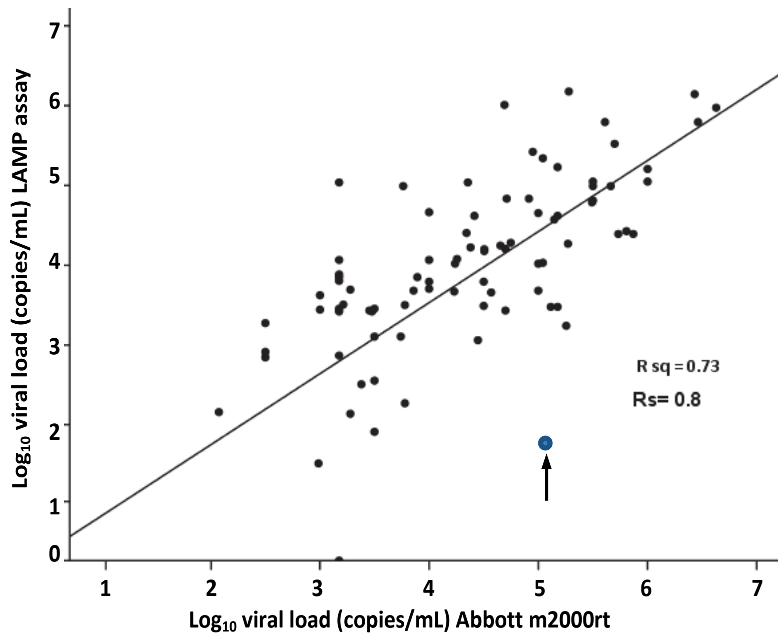


Figure 19. Correlation analysis for **Munich samples analyzed in Munich**. Results showed a direct linear correlation (R_s (92) = 0.8) between the viral loads obtained by Abbott m2000rt and LAMP assay. A regression analysis resulted in R^2 = 0.73 showing that ≥73% of the LAMP assay results fitted within the results obtained by the Abbott m2000rt assay. A sub-optimal quantification by LAMP assay (blue point shown by an arrow) was realized for sample V1221944.

4.3.3 Nucleic acid sequencing and primer modification.

4.3.3.1 Nucleic acid sequence analysis of primer binding sites of patient samples

Sequencing of samples with discrepant results between the LAMP and Abbott m2000rt systems was performed to check for any mutations or mismatches within the primer regions. A selected number of patient samples presenting more than $1\log_{10}$ deviation between the 2 assays, together with 2 more samples (NYK73501 and NYK73461) whose viral loads were within $1\log_{10}$ were selected and sequenced in the integrase gene region. The sequences generated were aligned with primer target sequences and analyzed for any base-pair mismatches. Results showed an average of 3 base-pair mismatches (Table 12) at the Forward outer primer (F3) target for all these samples. Within this F3 target, general mismatches of thymine (T) with guanine (G) at position 4730; guanine (G) and adenine (A) at position 4733 and thymine with cytosine (C) at position 4742, a second last position at the 3'end (Figure 20) were observed. The results further showed that the two samples (NYK73163 and V1221944), which despite very high viral loads obtained by Abbott m2000rt system assay recorded poor sensitivity in quantification by the LAMP assay, showed a high number of mismatches. It was further observed that these 2 samples had mismatches at every target of the essential outer and inner primers (except for B3 primer target which was not analyzed) which are necessary for the start of the amplification process. A further mismatch was recorded for sample NYK73163 at the last position of the 3'end of the inner primer (BIP) (Figure 20).

Although the sequence pattern for the reverse outer primer (B3) could not be generated due to sub-optimal amplification in the sequence reaction, a possibility of mismatches also at the B3 target for samples NYK73163 and V1221944 was suspected. This finding points towards the fact that mismatches in these two specific primers, mainly the F3 and B3 outer targets could be crucial in achieving a higher sensitivity for LAMP assay; in fact a mismatch at an outer target followed by another at an inner target has a potential to even reduce further the sensitivity of LAMP assay as seen with sample NYK73163. On the other hand, mismatches for NYK73501 and NYK73461 were located mainly in the middle part (thymine (T) and guanine (G) at position 4730 and guanine (G) and adenine (A) at position 4733). The only extra mismatch for sample NYK73501 was at the first position in the 5'end. Although sample V1221944 belonged to subtype C virus, there was a variation in its sequence patterns at B2 and B3 primer targets, where it showed several mismatches compared to other C subtypes sequenced. Sample NYK73163 on the other hand belonged to HIV-1 group M subtype A1. Although useful in speeding up the amplification process, the loop target primers, have not been found (by design) to be very important in the amplification of LAMP products. However, for the sake of a quantitative detection which estimates the viral load based on time, the importance of

these primers cannot be underestimated. In this study however, only a single mismatch was found for one LF target. Based on the position within LF, this single mismatch was deemed not to have any major effect on the sensitivity of LAMP, and as such may not have contributed much to the poor sensitivity shown for sample NYK73163. Based on the 10 samples analyzed, these results did not show a single pattern of mismatches among subtypes A1 and C identified (Table 12).

		Outer primer	**Inner primers**		**Loop Primers**		**Total number of mismatches**
Sample Number	**Subtype**	*F3*	*FIP (F1c+F2)*	*BIP (B2+B1c)*	*LF*	*LB*	
NYK73163	A1	3	3	4	1	0	**11**
NYK73528	A1	4	0	1	1	0	**6**
NYK73575	A1	3	1	1	1	0	**6**
NYK73621	A1	3	0	2	1	0	**6**
KEM6507	A1	4	1	2	1	0	**8**
RT2225	A1	3	1	3	1	0	**8**
NYK73501	C	3	1	2	1	0	**7**
NYK73461	C	2	1	2	1	0	**6**
KEM 6444	C	3	0	2	1	0	**6**
V1221944	C	3	0	15	1	0	**19**

Table 12. Number of mismatches for clinical samples showing >1$\log_{10}$ discrepant results between Abbott m2000rt and LAMP assays. Results showed samples NYK73163 and V1221944 (outliers shown in figure 17 and 19) to have the highest number of mismatches both for outer (B3) and inner (FIP and (or) BIP) primers. Samples NYK73528 and KEM6507 which had shown sub-optimal amplification also recorded a high number of mismatches at F3 outer region. The number of mismatches at outer B3 primer was not included in the analysis due to sub-optimal amplification at the B3 region. It was however suspected that the mismatches at B3 would not significantly alter the results obtained in the table.

4.3.3.2 Design of modified primers

Primer target F3 was modified to remove mismatches at positions 4730, 4733 and 4742 (Figure 20; F3 shown in red). The modified and the standard sets of primers were then used in re-analysis of samples and the results compared. Samples IIIB and MVP899-87 which had been used as standard subtype B samples, together with single samples each representing subtypes D, F and G were included in the analysis. The results obtained for the modified set showed a drastic reduction in the viral load quantification for the two subtype B viruses (Table 13), with sample MVP899-87 recording approximately 9.8×10^4 copies/mL by standard primer set against 1.3×10^1 copies/mL for modified primer set, while sample IIIB recorded approximately 8.8×10^4 copies/mL by the standard primer set against 1.3×10^2 copies/mL by the modified primer set. On the other hand, an improved sensitivity with the modified primers on samples NYK73528 and KEM6507, which had shown poor sensitivity

with the standard primer set, was obtained. Sample NYK73501 was still within the $1log_{10}$ range between the two primer sets which was a reproducible result from the initial analysis during the viral load quantification (Table 13). In general, the modified primer set showed an improved sensitivity among some HIV-1 M non-B subtypes as compared to the B subtypes where it had poor sensitivity. The drastic reduction in the viral loads of the two B subtypes with the modified primers points towards the effects of mismatches at the target primers on the sensitivity of LAMP assay.

With the modification of F3 outer primer target additional mismatches were introduced into the primer sequence of subtype B at the 3 positions (Figure 20). Since F3 target is important for initiating amplification, there was a possibility of non-optimal amplification initiation which may have been reproduced in the subsequent reactions. This observation is also supported by an improvement in the sensitivity of the assay for the 3 non-B subtype samples, albeit with a small margin. Since only the F3 outer target was modified, poor sensitivity still shown with sample NYK73163 may be as well attributed to further mismatches in other targets, namely the FIP and the B3. It is worth noting that FIP target had a similar number of mismatches as the F3 and the BIP targets, with BIP even having a mismatch at the last position on the 3'end, which could influence the efficiency of amplification. Although the nucleic acid sequence of B3 target was not completely achieved, it is possible that this target too had a similar number of mismatches as F3 for this sample. The increased number of mismatches at these sites among other factors is deemed to have influenced the sensitivity of LAMP assay on this sample, even with the modified set of primers.

Sample number	Viral load (copies/mL) - Abbott (m2000rt)	Viral load (copies/mL) LAMP	
	Viral Load	Standard primers	Modified primers
NYK 73163	1,300,000	0	0
NYK 73528	300,000	0	560
NYK 73501	60,000	21,000	9,000
KEM 6507	11,000	40	900
V1116227	22,000	25,000	800
V1216136B	110,000	50	2,600
V0634505	2,500	150	140
MVP899 - 87	2,900,000	98,000	10
HTLV-IIIB	2,100,000	88,000	130

Table 13. Comparison of viral load results for LAMP obtained by the standard and the F3-modified sets of primers. Results showed a drastic reduction in viral loads for subtype B samples (IIIB and MVP899-87) by the F3-modified primers compared to standard primers (red square box). A trend of improved sensitivity by F3-modified primers among 3 samples representing non-B viruses is also shown (blue square boxes).

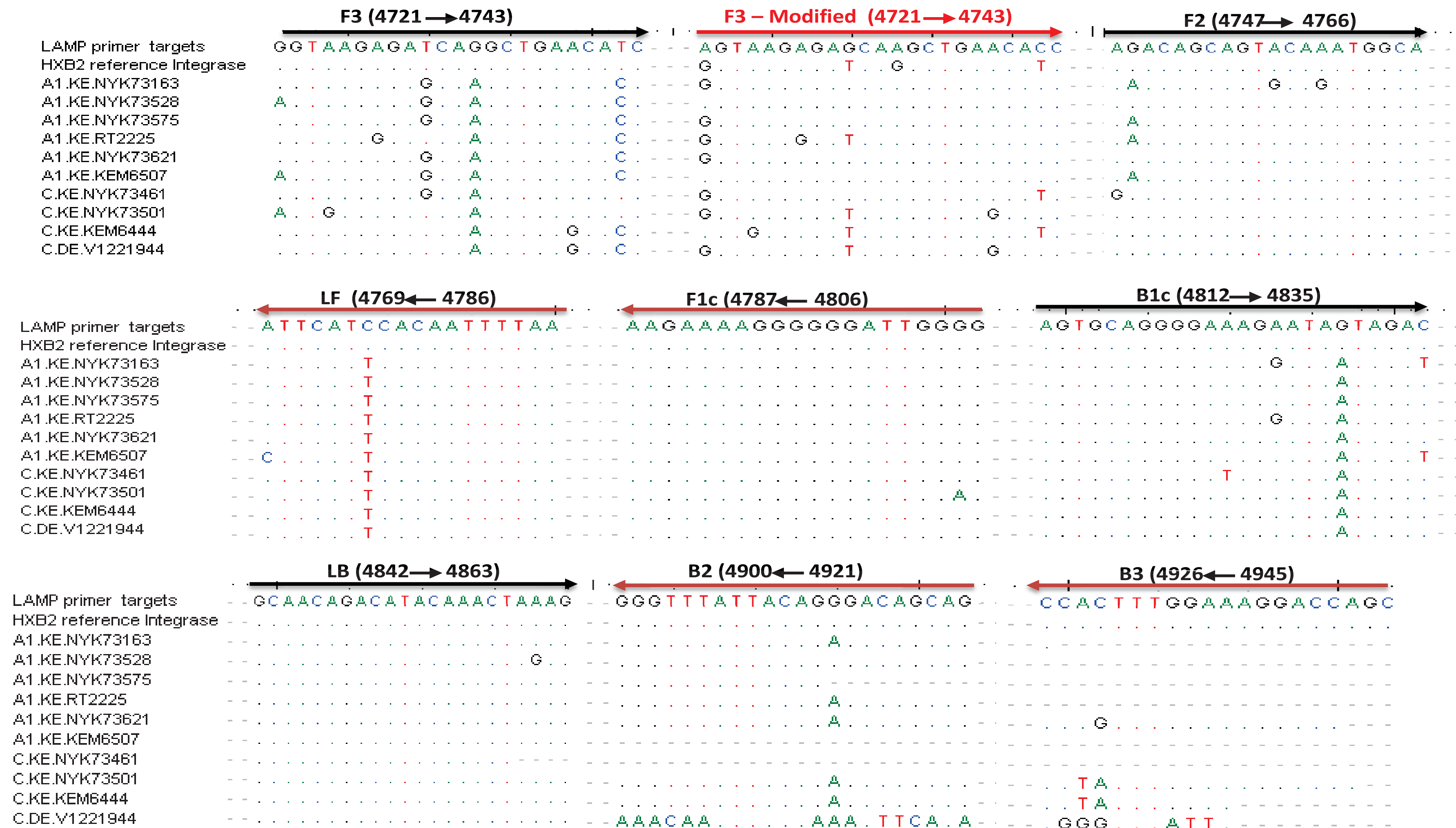


Figure 20. Nucleic acid sequence alignments of sequenced samples with HIInteg standard and F-3 modified primers. Results show the sequence patterns of both standard and F3-modified (red title) primers with modifications at positions; 4721, 4730 and 4742. A high number of mismatches is shown for samples NYK73163 and V1221944 which had shown poor sensitivity during amplification. The primers were re-tested with sequenced Kenyan samples including sample NYK73163 which had shown sup-optimal amplification by LAMP assay. F3: Forward primer, F2: Forward inner primer 2, LF: Loop forward, F1c: Forward inner reverse complement primer, B1c: Backward inner reverse complement primer, LB: Loop backward, B2: Backward inner primer, B3: Backward primer. FIP (F1c+ F2), BIP (B2 + B1c). Partial or no sequences at the outer prime B3 were obtained for some samples.

Chapter 5

5 Discussion

With the rising HIV drug resistance due to ART treatment in Sub-Saharan Africa and the possible transmission of ART resistant strains, a global threat to the success already achieved in HIV/AIDS control is eminent. Measures to curb this spread of resistant strains including an improvement in laboratory capacities and introduction of simple, affordable and rapid viral load measurement technologies should be put in place within these countries with limited resources. This study showed that using integrase primers, LAMP assay exhibits a potential for semi-quantitative detection of viral load of HIV-1 group M viruses in blood and plasma for treatment monitoring of HIV infected patients. The results in this study are considered not sufficient to allow quantitative measurement of viral load as recently reported using p24 primers (Zeng et al., 2014). Semi-quantitative detection has also been recently reported for microfilaria detection by LAMP (Drame et al., 2014) used to prevent serious adverse events before exposure to treatment.

5.1 Evaluation and testing of primers and assay conditions

5.1.1 Preliminary assessment of primers and assay conditions

In the first part of this study it was shown that the first four (outer and inner) primers designed for LAMP amplification to amplify HIV-1 templates in normal rt-PCR using Taq polymerase enzyme effectively worked for HIV-1 subtype B. Taq polymerase enzyme is important in PCR reaction due to its ability to withstand high temperatures in thermo-cyclic reactions. On the other hand Bst polymerase enzyme is important in isothermal reaction due to its ability to displace DNA strands under a thermo-stable condition. For this reason Bst polymerase remains an important enzyme to initiate amplifications in isothermal reactions such as LAMP. The specificity of these primers was ascertained by restriction digestion of LAMP amplicons which yielded the expected restriction products from the specific endonucleases. The finding that the selected primers were able to amplify cDNA templates at defined laboratory conditions for rt-PCR showed the possibility of using these primers not only for LAMP amplification, but also in rt-PCR. The findings thus depict rt-PCR as an alternative quality control assay for LAMP primers and would therefore be important as a troubleshooting tool in the event of challenges experienced during LAMP analysis.

As described and shown in previous studies (Mori et al., 2001; Parida et al., 2008; Tomita et al., 2008) this study revealed that all detection methods including, real time monitoring of turbidity, visualization of fluorescence under UV light and gel electrophoresis of LAMP amplification products

were equally reproducible. Additionally use of a HNB colour detection dye that neither requires real time monitoring nor UV lamp for detection was also effective and reproducible in qualitatively detecting the LAMP products (Figure 6). Use of HNB dye not only guarantees simplicity of the assay thereby reducing costs but also eliminates post amplification procedures which are time consuming and a risk to laboratory contamination by amplified products. Although T-test results comparing real time detection to HNB dye detection showed no significant differences in the means, results however showed a general lateness in time to threshold detection by visual colour change compared to real time detection by a turbidimeter. In their study Bista and colleagues (Bista et al., 2007) observed that the colour change for samples with high viral loads (>10^7 copies/ml) were detected earlier (i.e. <30 minutes), whereas those samples with viral loads between 10^4 and 10^5 copies/mL were detected after long (i.e. >60 minutes) incubation periods. The finding of late detections by visual colour change presents a negative implication if the visual detection method is to be used for a quantitative detection.. Indeed it was realized during this study that the colour change at the visual level indicated an all-or-none phenomenon implying a "yes" or "no" result and as such did not exhibit a quality for quantitative analysis. This finding thus presents a limitation of this assay, if visual detection is to be used as a quantification method since the HNB colour intensities between different viral loads would not be discriminated.

5.1.2 Stringency of primer binding

The second part of this study evaluated the primers designed for targets of 4 major conserved regions in the HIV genome: LTR (HiLtr), p24 (HiP) in the *gag*, integrase (HiInteg) in the *pol* and gp41 (HiGp) in the *env* gene. Selection of the most suitable primer set was important if it was to be used in testing samples from different HIV-1 group M subtypes. Indeed various studies have demonstrated that HIV genetic diversity pose a major problem in PCR quantification, especially among the non-B subtypes (Damond et al., 1999; Gueudin et al., 2007; Scott et al., 2009; Wirden et al., 2009). The first finding in this study demonstrated that the 4 sets of LAMP primers ably amplified HIV-RNA from cell culture supernatants samples belonging to subtype B. This finding offered the possibility of having a variety of options in the conserved regions of the HIV genome for selection and designing LAMP primers (Figure 7). Previous studies have also demonstrated that improved quantification results were achieved by use of dual-target assays (Pas et al., 2010), nevertheless in this study only one target was used.

5.1.3 Influence of mismatches

Even with the ability of the 4 primer sets to detect samples of subtype B virus, there was a variation in analytical sensitivity among them. HiInteg and HiP primers, targeting the integrase in the *pol* and

p24 in the *gag* gene respectively, showed higher analytical sensitivities compared to the remaining 2 primer sets, as was shown in Figure 7. The discrepancies among these primers could mainly be attributed to the number of mismatches within these primer sets with the subtype B virus upon which they were tested during quantification (Table 5). During the mismatch analysis of the various group M subtypes, the least average number of mismatches was realized with HiInteg primers followed by HiP primers. The two previously published primer sets had recorded the highest analytical sensitivities, detecting a virus concentration as low as 2.1×10^1 and 2.9×10^2 copies/mL respectively within a subtype B virus sample panel.

Detrimental effects of mismatches on nucleic acid amplification by PCR assays have been well elucidated (Klein et al., 2001; Whiley and Sloots, 2005) and although scarce, available publications have also shown that these mismatches have the potential to affect quantification of amplification products in real time PCR (Stadhouders et al., 2010; Whiley and Sloots, 2005). However it is worth noting that even with the high number of mismatches two sets of primers, HiP and HiLtr, not only detected all HIV-1 group M subtypes, but also group O while HiInteg detected only HIV-1 group M subtypes (Table 4). On the other hand HiGp only detected group M subtype B. The finding of detection of group O viruses by HiP and HiLtr despite the high number of mismatches may not be explained conclusively in this study. However it can be hypothesized that there is a probability that the detrimental effect of mismatches in LAMP primers are dependent on the 8 arbitrary regions (F3, B3, F2, F1c, B1c, B2, LF, LB) within the 6 targets and the positions of the mismatches within these regions (5'end or 3'end). Indeed several studies (Bru et al., 2008; Christopherson et al., 1997; Klein et al., 2001; Kwok et al., 1990; Stadhouders et al., 2010; Whiley and Sloots, 2005) have shown that mismatches at the 3' end region of a primer are known to exceptionally be detrimental to the efficiency of PCR amplification, which may not only lead to underestimation of the initial copy numbers but even to a complete abolishment of the amplification (Stadhouders et al., 2010). This assertion of position dependence was well elucidated in this study, when different nucleic acid sequences of subtypes of HIV-1 group M, O and HIV-2 were aligned with HiInteg primers.

As shown in Figure 11 (a and b) mismatches were realized at different positions of different targets within B and non-B subtypes. Mismatches within MVP899-87 which was a standard sample were realized at the second base from the 5'end within F3 with no mismatch at the B3 outer primer sequence targets. On the same sample, another mismatch was observed at the last base at the 3'end of B1c target and no mismatch at the B2 and the 2 FIP sequence targets of the primers. MVP899-87 had been quantified and was demonstrated to have the highest analytical sensitivity with HiInteg primer

set. The HiInteg primer set showed an average of 7 mismatches (Figure 11c) within the F3 target at different positions including the last position at the 3'end among the group O viruses, which it failed to detect. This finding is possibly a predictor of the importance of F3 and B3 sequence targets in the initiation of amplification and any mismatches at the 3'end of these targets are likely to affect the amplification process.

Detrimental effect of mismatches at F3 and B3 sequence targets could be supported by the observation that despite a mismatch at the 3'end of the B1c sequence target, the amplification and quantification was not affected for the MVP899-87 sample. Indeed F3 and B3 are also known as strand displacement primers and as such have their major role during strand displacement at the initiation of the isothermal reaction, whereas FIP and BIP have their major role in loop formation (Parida et al., 2008). It therefore means that a mismatch at the 3' end of F3 or B3 and also a mismatch at the 3'end of F2 and B2 are likely to be detrimental by lowering sensitivity and even leading to non-amplification in the LAMP assay.

The results obtained by other sets of primers as well show the possibility that mismatches at the 5' end and the internal region of LAMP primers at F3, B3, F2 and B2 may not have a major influence in LAMP amplification process, since all these primers were still able to amplify the templates. Results by these primers also showed that mismatches at the 3'end of F1c and B1c seemed not to have detrimental effects on the amplification process of the tested samples. Apart from the outer and the inner primers, studies have shown that loop primers although not very necessary in the initial LAMP amplification process, accelerate the reaction process by one third to one half of the reaction time (Nagamine et al., 2002; Parida et al., 2008). This added benefit of acceleration of the LAMP process by these primers underscores the need to ensure that only limited or no mismatches exist within the LF and LB primer sequences and if present they should be avoided at the 3'end. For the purposes of this study, loop primers are particularly essential since the estimation of viral load for HIV positive samples was based on the time to threshold detection.

Two primer sets HiInteg and HiP were therefore selected for further evaluation and refinement for analysis of clinical samples. Within these two sets of primers it was realized that HiP set of primers was very sensitive among the clinical samples but was associated with a low specificity.

5.1.4 False positive reactions in LAMP assay

The low specificity due to false positive results realized for the selected HIV negative plasma by HiP set of primers was an important finding in this study. Furthermore this primer set not only amplified

HIV-1 group O viruses but also HIV-2 viruses, despite the fact that HIV-1 and HIV-2 viruses have a restricted sequence homology in the p24 region. For a long time this sequence diversity has hindered the development of a viral load assay able to reliably quantify all subtypes of both viruses (Pas et al., 2010). Since false positive results were obtained for 4 HIV negative samples during analysis of plasma and dry blood spot, there was a high possibility that the results obtained for HIV-2 by the HiP set of primers were false positive. Some of the possible major reasons for false positive results would include sample contamination, cross-reactivity, mis-priming and primer dimerization. .

5.1.4.1 Sample contamination

One of the major draw backs in LAMP, just as in PCR, is the problem of carry over contamination which is mainly contributed to by the high sensitivity of the assay. Previous publications (Bai et al., 2011) have suggested that the products of LAMP amplification are very stable and as such not easily degraded, posing a risk of carry over contamination. In this study however, although at the initial stages contamination was a major setback, precautions were put in place which included not opening the tubes after amplification, apart from using different rooms, clean benches and gloves. In some experiments viral load samples (<5,000 copies/mL) were used to minimize the possibility of carry over contamination. During the analysis negative controls were used which comprised of water, Tris-buffer, poly A or 0.9% NaCl. The negative controls continuously tested negative. Therefore the false positive results realized by HiP primers during the study could not fairly be attributed to sample contamination. [1]

5.1.4.2 Cross-reactivity

The positive amplification of HIV-2 by HiP primers could alternatively have resulted from cross-reactivity, which in such a case in patient care would lead to misdiagnosis and inappropriate patient management. Cross-reactivity has previously been reported (Pas et al., 2010) for some viral load commercial assays. During this analysis, HiP primers were designed only for single targets within the HIV-1 M:B genome. Many commercial assays have currently introduced the concept of dual targets in an attempt to reduce the possibility of cross-reactivity with primers (Damond et al., 2010; Pas et al., 2010). The fact that only single target for HiP primers were available could be hypothesized for the false positive results, a matter that invites further evaluation.

[1] To further rule out sample contamination, repeat tests were performed with HiP primers after 6 months using fresh aliquots of cell culture supernatant samples. False positive results were still generated. HIV-1 group O and HIV-2 showed false positive results in the presence or absence of reverse transcriptase enzyme. With these latest results a possibility of master-mix or carry over contamination was ruled out.

Further reasons for false positive results in nucleic acid amplification systems include the presence of primer-dimer formations as a result of extensions of self-annealed primers. Not many publications have dwelt on the effect of primer-dimer formation on LAMP and since the subject was not part of the study, it was not possible to elaborate the effects of primer-dimer formation. Although at the primer level, a possibility of primer-primer binding within FIP and BIP primers of HiInteg (Table 1) leading to primer-dimer formation would have been anticipated, the results generated by HiInteg set did not, however, show the challenge of false positive results.

5.1.4.3 Mis-priming

Mis-priming is another possible reason for false positive results in PCR amplification. Since within the human genome also exist retroviral elements, these elements have the potential to bind to primers designed to detect retroviruses. Briefly, as already mentioned in the literature review, HIV belongs to the family *Retroviridae* and during co-evolution between viruses and humans, many retroviruses tend to "capture" bits of the human genome and likewise "deposit" bits of their genomes in the human genome. Therefore primers designed to detect HIV could in some cases share significant homology within the human genome structure and lead to false positive results. The argument of existing homology between the human and HIV genomes would thus seem plausible in respect to the results obtained in this study where false positive results mainly occurred after 30 minutes and were only seen with negative plasma samples and not in saline used as negative controls. The argument may further be strengthened by the high number of primers used during LAMP amplification assay which increases the risk of binding to non specific targets as opposed to normal or quantitative PCR. Furthermore, HIV-1 group O and HIV-2 detected by HiP set of primers had been cultivated in the human H9 cell line and as such the potential of traces of human DNA within the cell line supernatant can not be ruled out.

The finding in this study of false positive results for HiP primers occurring after 30 minutes, would point towards a possibility of specific binding of LAMP primers to unspecific target sequences in the sample. One reason may be due to the complex cycling process of LAMP (Figure 2b), since amplification mainly depends on loop formation and amplification of these loops leading to rapid formation of pyrophosphate deposits. The latter observation would therefore imply that an extension of reaction time would potentially lead to unspecific amplifications. As was also realized in this study, analysis of LAMP products by gel electrophoresis was shown to be highly sensitive and specific and as such could be used to clarify the reason for unspecific amplification. However this study also realized occasional atypical banding patterns for HIV negative samples following

amplification (Figure not shown). These banding patterns in the absence of nucleic acids have previously been reported in different studies (Inacio et al., 2008; Kuboki et al., 2003; Teng et al., 2007; Yeh et al., 2005). The challenges of atypical banding as well as potential high risk of contamination pose a significant hurdle in using gel electrophoresis. Furthermore gel electrophoresis would increase the amount of time for LAMP by an extra 1½ hours and requires highly sophisticated equipment in laboratories.

In their study, Curtis and colleagues (Curtis et al., 2009) also showed the presence of false positive results by a similar set of p24 target primers in a LAMP reaction. Zeng and colleagues, however, in their recent publication showed quantitative detection of HIV-1 virus by LAMP assay using the p24 target primers (Zeng et al., 2014) without reporting any false positive results in their evaluation. The primers used by Zeng´s group and those used in this study mainly differed in their positions within the *gag*-p24 target of HXB2 genome (1224 – 1441 for Zeng´ versus 1311 – 1515 used in this study). Alignment of the two sets of primers with HIV-1 group M (A to F) subtypes (Appendix 1 and 2) however showed that the p24 (HiP) target primers used in this study (Appendix 2) exhibited a high specificity among the different non B subtypes compared to those of Zeng´s group (Appendix 1). It is however worth noting that the primers designed by Zeng´s group mainly targeted HIV-1 group M subtypes B, C and BC circulating in China.

5.1.5 Visual detection of LAMP amplification products

One of the advantages cited for LAMP assay is the ability to visually detect amplification products by colour change due to the presence of intercalating dyes and by turbidity due to accumulation of magnesium pyrophosphate deposits. The dyes however are not sequence-specific and as such bind to DNA indiscriminately including non-specific amplifications. On the other hand, use of turbidity may not be reliable as magnesium pyrophosphate will also accumulate during non-target specific amplifications. This challenge would possibly be overcome by including fluorescence labeled primers and quencher probes into the reactions. Fluorescence labeled primers would then incorporate only into target specific amplicons and the quencher probes quench any unincorporated labeled primer allowing the detection of only specific amplification products. The final result would therefore lead to visual distinction between target specific and non-specific amplifications, since fluorescent signals would only be generated for the specific targets. Replacement of turbidity as the final amplification signal by using fluorescence labelled primers has successfully been used in sequence specific detection by LAMP assay (Curtis et al., 2009). In a recent publication for real time detection of a

HIV-2 integrase target by LAMP, a Hex fluorescent label and Black Hole Quencher were effectively used to quantify HIV-2 group A and B virus (Curtis et al., 2014).

5.1.6 Sensitivity of LAMP assay and establishment of detection limits

5.1.6.1 Sensitivity of LAMP using HIV cell culture supernatants

Analysis of HIV cell culture supernatants of various subtypes showed a general lower sensitivity of LAMP assay compared to the Abbott m2000rt system, since only 60% of the samples fitted within $1\log_{10}$ difference (Figure 15). Some of the samples presenting $\geq 1\log_{10}$ were dilutions obtained from samples with higher viral loads measured by the Abbott m2000rt system. These samples were measured in triplicate in the LAMP assay. There is thus a theoretical possibility that inconsistencies could have occurred during the dilutions. In general it has been established that errors including pipetting errors, freeze thawing cycles and handling steps could detrimentally affect a test thus leading to lower quantifications. It is therefore possible that diluting samples before testing on LAMP assay could lower the analytical sensitivity of this assay. However the fact that a correlation of 70% was realized between LAMP assays (established as semi-quantitative) and Abbott m2000rt system (purely quantitative assay) presented a promising potential use of the LAMP assay in the estimation of viral load for undiluted clinical samples.

5.1.6.2 Establishment of LAMP detection limits

Two fundamental achievements of this study were the establishment of lower threshold limits and the generation of a mathematical model for estimation of the viral load by LAMP assay. The achievement of LLOQ 9.8×10^3 (approximately 10,000) copies/mL basically means that the LAMP assay should detect all HIV-1 group M positive samples ≥10,000 copies/mL within the first run. LAMP assay was further able to detect >70% of samples with viral loads >3,000 copies/mL, apart from the LLOD which was established at a 50% detection rate at a viral load of 1.2×10^3 copies/mL.

Although samples >1,000 copies/mL were theoretically meant to be detected within the first 45 minutes within the first run, the majority of samples with viral loads <2,500 copies/mL were only detected within 2 runs, especially as the viral loads approached 1,000 copies/mL. This finding was a useful indication of a potential limitation of LAMP assay in testing viral load especially in patients on treatment with viral load levels close to or below 1,000 copies/mL.

The current WHO guidelines (World Health Organization, 2013a) recommend the detection of viral load ≥1,000 copies/mL after 2 consecutive tests in order to identify a treatment success or failure. This new recommendation is a lower threshold compared to the recommendation made in 2010 which

had put the treatment failure at viral load measurement ≥5,000 copies/mL (World Health Organization, 2010a; World Health Organization, 2010b). Therefore when using an optimized LAMP assay to meet the new WHO threshold, samples without a signal (undetected) within the first 45 minutes in the first run should be retested and in case of discordant results a 3rd test would be necessary. Some studies (Curtis et al., 2008) have also realized that doubling the reaction mix and the template volume increases the sensitivity of the LAMP assay within samples of low viral loads. This assertion however needs further investigation for HIV clinical samples between 1,000 and 10,000 copies/mL for non-B subtypes. An earlier attempt during this study to improve the sensitivity of the assay by increasing the buffer concentration of the master mix did not yield any improvement.

During this study it was also realized that LAMP would cost approximately € 8.00 (US $ 11) per test as compared to € 38.00 (US $ 52) per test for Abbott Real Time HIV-1 assay. This calculated cost would therefore mean that any 2 extra tests performed by LAMP assay would still be cost effective compared to the price of commercial viral load assays currently available.

5.2 Analysis of clinical samples

5.2.1 Optimization of sample material for LAMP analysis

Another objective of this study was to analyze various categories of samples by the LAMP assay to identify the most appropriate sample material that would be simple, cost effective and stable for viral load quantification by the LAMP assay. As revealed in this study, extracted nucleic acid provided the most reliable results, with extracted whole blood and plasma providing optimal results. Results with dry blood spot extracts could not reliably be interpreted quantitatively as the results obtained with these extracts showed overestimations and underestimations compared to established viral load measurements by the Abbott m2000rt system (Figure 18).

5.2.1.1 Dried blood spot samples

Dried blood spots have currently been used for HIV-1 RNA quantification, DNA genotyping and other diagnostic assays. They have particularly been used as an alternative to peripheral blood plasma for periodic viral load testing (Alvarez-Munoz et al., 2005; Bertagnolio et al., 2010; Bertagnolio et al., 2007; Garrido et al., 2009) especially in resource limited countries where they have been found to perform equally well as plasma (Johannessen et al., 2009). The advantages of using DBS are: **(i)** easy collection and storage as they neither require special collection equipment nor refrigeration **(ii)** requirement of a small volume of blood thus have been used in infants **(iii)** ease of transportation since they can be shipped as non-dangerous goods and **(iv)** the possibility of having extra samples for future analysis as each card takes about 5 to 6 spots (Bertagnolio et al., 2007; Brambilla et al., 2003;

Johannessen et al., 2009). All these qualities make DBS ideal for resource limited countries, and if used together with the LAMP assay the cost of viral load testing would significantly be reduced. Indeed some studies have estimated a cost reduction of U.S $ 2.67 per test (Neogi et al., 2012) when DBS are used instead of direct plasma for viral load testing. The results in this study however showed that there is a further need for improvement and optimization of LAMP assay if DBS samples are to be used.

5.2.1.1.1 Viral load analysis for LAMP using DBS samples

5.2.1.1.1.1 Sensitivity, specificity and correlation analysis

Although lower compared to those generated for plasma samples, the sensitivity of 76% and specificity of 77% reached for DBS samples offered a potential for improvement of LAMP for use with such samples. Even with promising results on sensitivity and specificity, no correlation between LAMP and Abbott m2000rt systems was achieved for DBS samples analyzed. The Spearman's correlation coefficient of 0.2 achieved within this group of samples was considered far too low to be reliable (Figure 18). This finding has the implication that LAMP as it is designed in this study cannot be used with DBS samples for determination of viral load.

5.2.1.1.1.2 False negative results

A closer look at the false negative results showed that although the majority of samples had viral loads <5,000 copies/mL by Abbott m2000rt system, 3 samples were >10,000 copies/mL. The corresponding plasmas for the 3 latter samples had been detected by the LAMP assay. This study had established a LLOQ of approximately 10,000 copies/mL and as such it was expected that the 3 samples would easily be detected by LAMP assay. The reason for failing to detect the 3 samples cannot conclusively be explained. One prominent reason might be insuffient extraction of HIV-RNA.

Viral loads for the remaining LAMP assay negative samples were <3,000 copies/mL, which points towards the need to optimize the extraction criteria for DBS samples. During extraction of these samples, one spot (70 µL), compared to 200 µL for plasma, was diluted in 1,000 µL extraction buffer, from which only 200 µL was used for extraction of the nucleic acid giving a reduction in the amount of viral load by >75%. The nucleic acid was eluted in 50 µL of elution buffer from which only 5 µL of the extract was finally used in LAMP analysis. Therefore only about 2% of the original sample viral load in the DBS was recovered for quantification by the LAMP assay. Other studies have also shown low sensitivity of DBS samples among patients with viral loads <5,000 copies/mL (Barin et al., 2005; Hamers et al., 2009; Scott et al., 2009; Uttayamakul et al., 2005). Perhaps evaluating the performance of LAMP assay by extracting DBS in 500 µL or 200 µL would yield better results.

5.2.1.1.1.3 False positive results

Specificity of LAMP for DBS samples was reduced (Table 10) by the presence of false positive detections. Furthermore some samples showed unreliably high viral load estimations compared to the results obtained by the Abbott m2000rt system. Even with the advantages of DBS cross-contamination is still a potential risk mainly during punching of the spots. Extraction of the DBS to generate maximum possible DNA or RNA material is also laborious and time consuming, potentially increasing the risk of contamination. Despite the precautions put in place, this study as well cannot rule out contamination as one possible contribution towards false positive results generated, since extraction and processing of these samples was performed manually. Even with avoidance of contamination, the presence of proviral DNA among DBS samples might be a major hindrance in correctly quantifying viral load values (Johannessen et al., 2009) and as such could have contributed to extremely high values as well as some false positive results. DBS consists of whole blood and cell associated HIV-1 DNA, which is present in the peripheral blood mononuclear cells. As a result, the cell associated HIV-1 DNA is frequently found in the extracted nucleic acid (Bruisten et al., 1993). Other studies have also reported abnormally high viral load measurements and false positive results attributed to the possible influence of proviral DNA (Johannessen et al., 2009; Waters et al., 2007). Although the presence of proviral DNA is beneficial in routine qualitative diagnosis (Curtis et al., 2008), its presence might become detrimental in viral load quantification.

Proviral DNA has been found to remain stable even with the fluctuation and reduction of plasma RNA below the detection limit (Lillo et al., 2004). This stability of proviral DNA would not be beneficial to the viral load quantification of patients, since constant detection of proviral DNA would mean that recovering patients would unnecessarily be switched to second line treatment thereby putting pressure on the available drug regimen. Further evaluation of DBS and their possible use with the LAMP assay should therefore be undertaken to address the challenges of unreliable results. In evaluating DBS for use in the LAMP assay, it would also be prudent to evaluate the performance of LAMP on DPS as well as dry serum spots since other groups have found these to be efficient in quantitative analysis and genotyping (Dachraoui et al., 2008; Plantier et al., 2005)..

5.2.1.2 Unprocessed whole blood and plasma

Although some studies have reported success with LAMP assay for unprocessed samples simply by heating plasma or whole blood (Bista et al., 2007; Curtis et al., 2008) or by directly using lysis buffer hence avoiding the heating step (Curtis et al., 2009), experiments in this study did not succeed in generating plausible results for samples treated by any of the two methods. However when the same

samples were processed and nucleic acid extracted from them, positive detections were made by the LAMP assay.

5.2.2 Performance characteristics of LAMP assay for HIV-1 virus positive plasmas

5.2.2.1 Sensitivity and specificity

This study depicts LAMP as a potential tool for use in quantifying viral load among patients of HIV-1 group M subtype B and non-B subtype infections circulating in Kenya. Analysis among German samples showed a sensitivity of 93% and specificity of 100%, whereas sensitivity between 82% and 86%, with specificity between 99% and 100% was achieved for Kenyan samples. The high sensitivities and specificities depict this assay to have a high property of detecting HIV-1 in clinical plasma samples, with low false positive results. Increased sensitivity of 95% and 100% which were achieved by this assay for samples with viral loads >5,000 copies/mL (Table 11) showed an increased chance of using LAMP to detect patients either failing treatment or those with putative HIV resistance during ART treatment. This would be in line with the earlier WHO recommendation (for resource limited countries) that continuous detection of values ≥5,000 after 3 consecutive tests following treatment initiation would indicate treatment failure (World Health Organization, 2010a). Upon improvement, LAMP assay would be able to identify patients with continuous viral load measurements >1,000 copies/mL as currently recommended by WHO for confirming treatment failure (World Health Organization, 2013a).

5.2.2.2 Correlation analysis

High correlation ≥0.8 achieved for all plasma sample categories between LAMP and Abbott m2000rt systems are an indication that the LAMP assay could potentially be optimized and used for estimation of viral loads in resource limited settings with limited deviations from the current commercial assays in use. Indeed average differences in viral load quantifications were calculated at $0.4\log_{10}$ for samples from Kenya and $0.3\log_{10}$ for samples from Germany (Table 8). In most cases the Abbott m2000rt system recorded higher viral load values compared to LAMP assay. The difference in $\log_{10}$ values was however within $1\log_{10}$ range hence indicating a higher correlation between the two assays. The findings thus show that although there is a wide difference in the limits of detection (≥50 copies/mL versus ≥10,000) owing to the differences in their test sensitivities, the two assays would potentially show minimal differences in detecting samples with higher viral loads. A closer look at individual $\log_{10}$ differences showed that samples with lower viral loads and those samples of non-B subtypes showed higher log differences between the Abbott m2000rt system and the LAMP assay, indicating that the sensitivity of LAMP, apart from viral load, is also subtype dependent (Figure 15). The

assertion of subtype dependence was also exhibited by a Kenyan sample (NYK73163) which could not be quantified by LAMP assay, despite the fact that a viral titer of >10^6 copies/mL was detected with the Abbott m2000rt system (Figure 17) and additionally a German sample (V1221944) which showed sub-optimal quantification (Figure 19).

5.2.2.3 Subtype specificity of RNA quantification by LAMP

Subtype dependence on the sensitivity of quantitative assays has been described previously and is not only restricted to the LAMP assay. Reported failures by commercial assays to monitor viral loads in patients infected by non-B subtypes have been documented (Alaeus et al., 1997; Damond et al., 1999; Gueudin et al., 2007), yet even others have reported under-quantification of viral loads among different commercial assays (Foulongne et al., 2006; Sloma et al., 2009; Wolff and Gerritzen, 2007). In their evaluation of three commercial assays Katsoulidou and colleagues (Katsoulidou et al., 2011) showed that $\log_{10}$ differences in viral loads were dependent on a specific HIV-1 group M subtype. Their observation could be partly attributed to the fact that the current commercial assays are predominantly designed based on highly specific primers targeting selected regions of the HIV-1 M:B genome and hence any mismatches within primer or probe binding sites would potentially have a significant influence on detection and accuracy of the particular test system.

The two samples that were under-quantified (Figure 17 (in red) and Figure 19 (in blue, pointed to by an arrow)) originated from Kenya and Germany. Contrary to expectation of a subtype B virus, the German sample was identified to belong to subtype C, whereas the Kenyan sample belonged to subtype A1. As already discussed on primer-target mismatches, a number of mismatches were found for these samples mainly at the primer 3'end. The Kenyan sample (NYK73163) for example, was found to have mismatches at or around the 3'end on the outer and the inner LAMP primers (Figure 20) an indication that mismatches at these positions within these primer regions would drastically affect efficiency of LAMP quantification. These samples were however amplified by the Abbott m2000rt system, probably due to the fact that the reagents, cycling conditions and primer/probe design have been optimized for mismatch tolerance (Scott et al., 2009; Swanson et al., 2006; Tang et al., 2007) even though the primers for the Abbott system (Tang et al., 2007) just like for LAMP assay were designed from the highly conserved integrase (IN) region. However, even with the high optimization, the Abbott m2000rt system has been reported even to miss a non-B sample from Greece (Katsoulidou et al., 2011).

5.2.2.4 Optimization of primers for improvement in LAMP quantification

With the finding of a possible detrimental effect of mismatches, designing primers for HIV-1 group M non-B subtypes remains crucial if LAMP assay is to be used for monitoring patients in Africa and other resource limited settings. Due to genomic strain variability of different subtypes of HIV-1 (Foley B. et al., 2013; Wain-Hobson, 1992) it is still not possible to develop universal primers and probes. Further, most of the available sequences in the sequence banks for primer and probe selection have mainly been for subtype B with non-B representing a minority, hence the risk of mismatches at the primer target sites are higher with non-B subtypes (Katsoulidou et al., 2011). In order to reduce the risks and effects of mismatches if LAMP assay is to be used for quantification, knowledge of all circulating subtypes would therefore be necessary, and perhaps designing a mixture of LAMP primers from consensus sequences of the circulating non-B subtypes would improve sensitivity of the assay.

5.3 Conclusion

The benefits of viral load testing for monitoring efficacy of HIV treatment, adherence of patients or early detection of resistance have been well documented in various studies. The high costs and limited use of the currently available commercial viral load tests expose the global community to a potential threat of circulation and spread of HIV resistant strains. If this potential threat is not controlled, a complete reversal of the gains realized so far in the fight against HIV/AIDS, especially in Africa is eminent. This study has managed to evaluate and establish a LAMP assay as a semi-quantitative procedure to measure HIV-1 group M viral load. The assay is thus shown as a potential alternative to monitor success of ART treatment especially in resource limited settings where low costs and short time to provide results are a necessity. Patients showing viral loads >5,000 copies/mL would easily be detected within 3 consecutive tests. With improved sensitivity detection of a majority of samples within the recommended >1,000 copies/mL range would be achieved. Present challenges realized in this assay which include potential primer mismatch, the problem of false positive results, sub-optimal analytical sensitivity for viral loads <5,000 copies/mL, insufficient detection from DBS and low subtype specificity need further improvement and evaluation.

5.4 Recommendations for improvement

Apart from the recently published studies for HIV-1 subtype quantification within a Chinese population (Zeng et al., 2014) and HIV-2 (Curtis et al., 2014), this is the first study attempting to establish LAMP for semi-quantification of HIV-1 in a limited resource country, where challenges of circulation of several HIV-1 non-B subtypes are expected. In order to address the challenges identified during the investigations, it is recommended designing LAMP primers from consensus sequences of most of the circulating subtypes, use of fluorescent labeled primers and quencher probes

to improve the specificity of LAMP detection, and parallel testing of samples or doubling the reaction mix to capture low viral load samples (<3,000 copies/mL). Further research is needed on the possible use of dry blood spots, plasma spots or serum spots. There is also need for future adaptation of the LAMP assay for detection of HIV-1 group O virus.

Finally, for the past decade African and other resource limited countries have focused their attention exclusively on scaling up HIV treatment and on prevention strategies. Little attention has been paid to monitor the success of treatment which in some cases has led to unnecessary switch to a second line treatment, drastically increasing the cost of treatment. Currently, the risk of exposure to resistant HIV strains is eminent due to a possibility of spread of resistant strains within these regions. Both factors strengthen the need to introduce a viral load monitoring tool which would be effective and efficient in HIV quantification as well as cost effective. LAMP assay upon improvement presents an opportunity as a cost-effective, rapid to run and user friendly viral load monitoring tool and thereby supporting optimal treatment of HIV patients in resource limited countries.

5.5 Summary

- The study established loop-mediated isothermal amplification (LAMP) assay for semi-quantitative detection of HIV-1 group M viruses using integrase gene target primers. There is need for further optimization among HIV-1 group M non-B subtypes.
- The assay as established is not able to detect HIV-1 group O or HIV-2 virus.
- Reliable quantification of LAMP assay is reduced for HIV-1 samples of viral loads <5,000 copies/mL
- Refinement of the assay is needed for dry blood spots in order to avoid a high rate of false positive and false negative results.
- Advantages of LAMP include, isothermal amplification hence it can be used in the field, short time to get results and cost-effectiveness making it suitable for resource limited settings.

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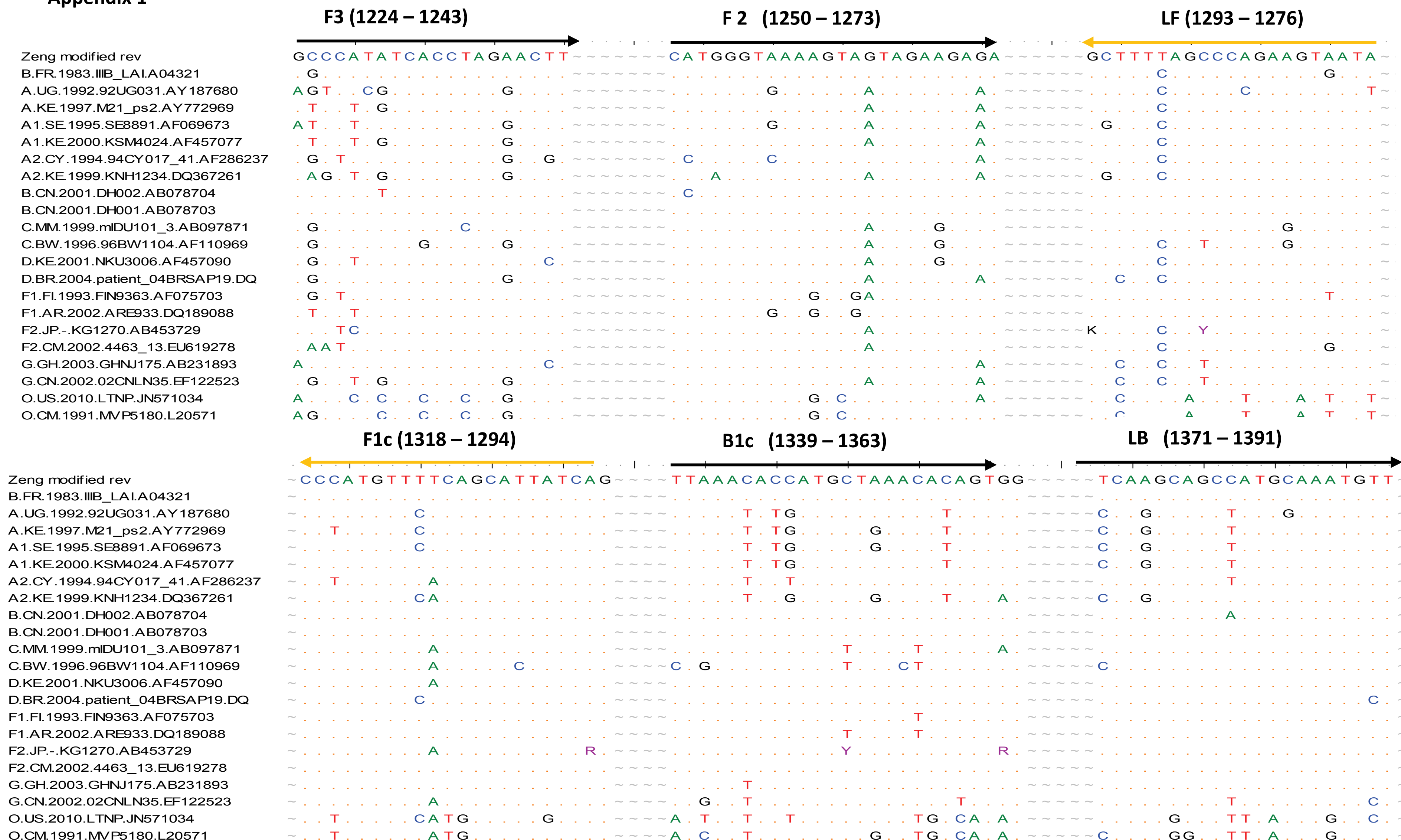
Appendix 1



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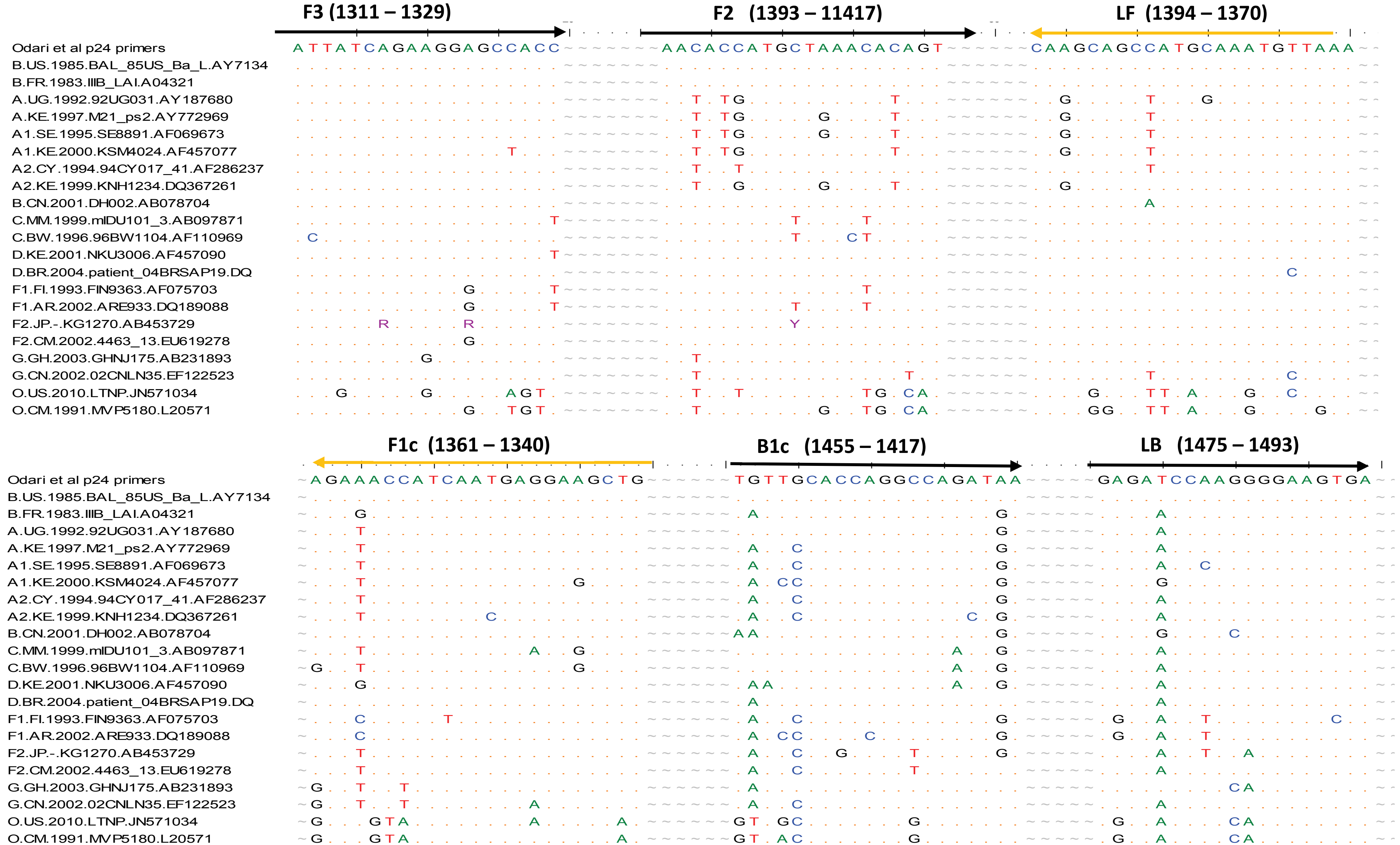
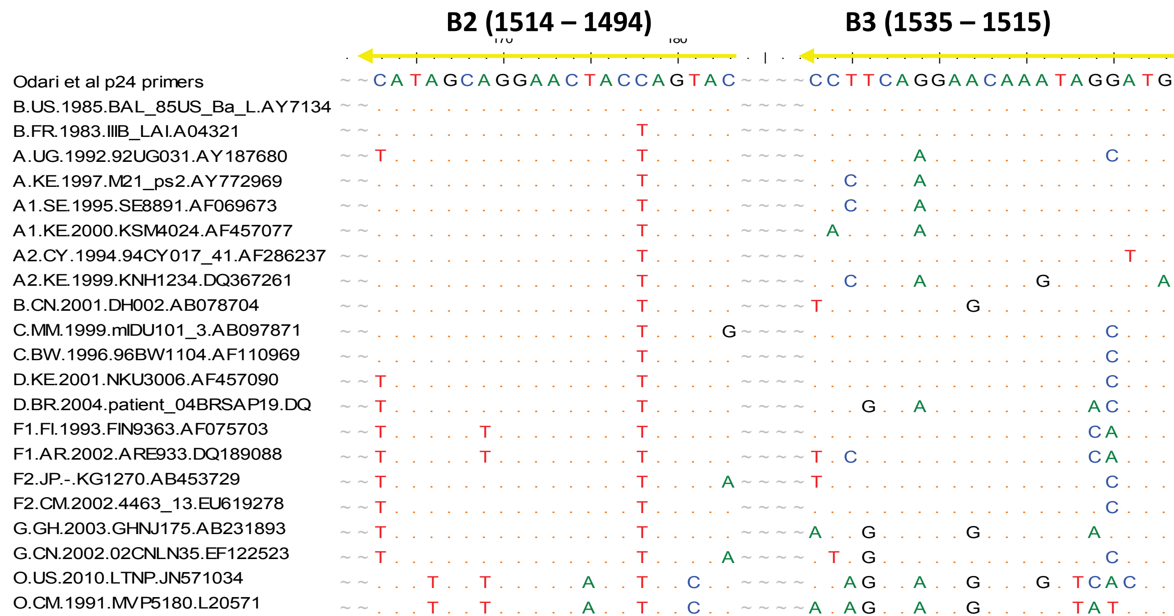
                                      B2 (1418 – 1400)                 B3 (1441 – 1420)
Zeng modified rev                 ~~~~CCATCAATGAAGAAGCTGC~~~~~~~GAATGGGATAGATTACATCCAG
B.FR.1983.IIIB_LAI.A04321         ~~~~..........G........-~~~~~~............G.G.......
A.UG.1992.92UG031.AY187680        ~~~~..........G........-~~~~~~........C..GAC.......A
A.KE.1997.M21_ps2.AY772969        ~~~~..........G........-~~~~~~........C..G..........
A1.SE.1995.SE8891.AF069673        ~~~~..........G........-~~~~~~........C..G..........
A1.KE.2000.KSM4024.AF457077       ~~~~..........G..G.....-~~~~~~........C..GG.........
A2.CY.1994.94CY017_41.AF286237    ~~~~..........G........-~~~~~~........C..GG.........
A2.KE.1999.KNH1234.DQ367261       ~~~~.......C..G........-~~~~~~....A...C.............
B.CN.2001.DH002.AB078704          ~~~~..........G........-~~~~~~..............G.......
B.CN.2001.DH001.AB078703          ~~~~..........G..G.....-~~~~~~..............G.......
C.MM.1999.mIDU101_3.AB097871      ~~~~.............G.....-~~~~~~......................
C.BW.1996.96BW1104.AF110969       ~~~~..........G..G.....-~~~~~~...........G..........
D.KE.2001.NKU3006.AF457090        ~~~~..........G........-~~~~~~...........GC........A
D.BR.2004.patient_04BRSAP19.DQ    ~~~~..........G........-~~~~~~...........GC.........
F1.FI.1993.FIN9363.AF075703       ~~~~....T.....G........-~~~~~~........C.............
F1.AR.2002.ARE933.DQ189088        ~~~~..........G........-~~~~~~........C.............
F2.JP.-.KG1270.AB453729           ~~~~..........G........-~~~~~~........C..G..........
F2.CM.2002.4463_13.EU619278       ~~~~..........G........-~~~~~~........C..G..........
G.GH.2003.GHNJ175.AB231893        ~~~~.T........G........-~~~~~~..G.....C..G.........C
G.CN.2002.02CNLN35.EF122523       ~~~~.T.................-~~~~~~..G........GC........C
O.US.2010.LTNP.JN571034           ~~~~TA..............A..-~~~~~~..C.........ACT..C...C
O.CM.1991.MVP5180.L20571          ~~~~TA........G.....A..-~~~~~~............ACT......C
```

Appendix 1: Primer alignment of sequences for the p24 target primers used in the Chinese study (Zeng et. al., 2014). Sequence alignment shows an average of 4 and 3mismatches at the F3 and F2 targets respectively for the non-B subtypes. This primer set is potentially specific for HIV-1 group M subtype B virus.

Appendix 2





Appendix 2: Primer alignment of sequences for the p24 target primers used in the current study (Curtis et al., 2008). Sequence alignment shows an average of 1 and 3 mismatches at the F3 and F2 targets respectively for all HIV-1 group M subtypes aligned. Although this primer set would potentially amplify all the subtypes aligned and potentially amplify some HIV-1 group O viruses, there is a possibility of sub-optimal amplification of some C and D subtypes due to the position of the mismatches.