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REVERSE TRANSCRIPTION LOOP MEDIATED ISOTHERMAL AMPLIFICATION FOR LOW COST HIV-1 VIRAL LOAD QUANTIFICATION IN RESOURCE LIMITED SETTINGS

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree

of

Master of Science in Medicine

Johannesburg, 2014

DECLARATION

I, Andrea Olga Papadopoulos, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

…….. day of ……. 2014

Dedication

To my mom and writer of non-science but not nonsense, Heidi Papadopoulos.

Publications and presentations arising from this study

Patent: Penny CB, Papadopoulos AO, Evans DH, Inventors. University of the Witwatersrand, applicant. Method for Testing HIV Viral Load. South African Provisional Patent Application P2517ZA00. 2012 September 18.

Patent: Penny CB, Papadopoulos AO, Evans DH, Inventors. University of the Witwatersrand, applicant. Method for Testing HIV Viral Load. South African Provisional Patent Application P2294ZA00. 2011.

Poster: A Colorimetric HIV-1 Viral Load Assay Based On RT-LAMP for Disease Progression Monitoring in Resource Limited Settings. In: Penny C, McNamara L, Evans D. 30 Years of HIV Science Symposium; 2013; Paris.

Poster: Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for Low Cost HIV-1 Viral Load Monitoring in Resource Limited Settings. In: Penny C, McNamara L, Evans D. South African Society for Biochemistry and Molecular Biology (SASBMB); 2012; Drakensberg.

Poster: Hydroxy-naphthol blue for quantitative detection in a reverse transcription loop mediated isothermal reaction. In: Penny C, McNamara L, Evans D. Wits University Faculty of Health Sciences Research day; 2012; Johannesburg.

Poster: Hydroxy-naphthol blue for quantitative detection in a reverse transcription loop mediated isothermal reaction. In: Penny C, McNamara L, Evans D. Molecular Biology Research Thrust (MBRT) for Wits University; 2012; Johannesburg.

Abstract

Background: A novel, isothermal nucleic acid amplification method, RT-LAMP, presents potential for nucleic acid amplification-based diagnostics in resource-limited settings. Low-cost HIV-1 viral load monitoring will improve access to ART for HIV-1-infected individuals present in settings where on-site viral load testing is unavailable.

Aim: The aim of this dissertation was to develop an RT-LAMP HIV-1 viral load assay by combining the RT-LAMP reaction with colorimetric amplification detection by hydroxy-naphthol blue dye.

Methods: Different approaches for HIV RNA extraction from patient plasma and culture supernatant were studied to obtain template for RT-LAMP. Reaction products for 4 different RT-LAMP primer sets were analysed using agarose gel electrophoresis and restriction digestion.

Results: The first 3 primers sets produced persistent off-target amplification. The fourth primer set, designed against culture supernatant DU179, produced a target-specific colour change from violet to blue after 1 hour, following optimisation of amounts of Mg_2SO_4 and AMV RT. Further studies showed HNB detection sensitivity to template copy number.

Conclusions: Initial reaction conditions pertaining to an RT-LAMP based, colorimetric HIV-1 viral load assay were established. Further work is required to determine the reaction duration at which the colour change represents a viral load of ≥1000 copies HIV RNA per ml plasma.

Acknowledgements

Dr. Clem Penny for supervision of the project.

Dr. Denise Evans for supervision of the project, co-ordinating whole blood sample collection as well as organising financial assistance through the Health Economics and Epidemiology Research Office (HE_2RO).

Ms. Lynne McNamara, for providing frequent, detailed technical advice in all molecular aspects of the project along with morale encouragement and support.

The Department of Internal Medicine for the use of their facilities at the Medical Oncology Research Laboratory where the majority of the research took place.

The Phlebotomists of the CHRU, Right to Care, iThembaLethu Clinic, Helen Joseph Hospital, Johannesburg for whole blood collection and informed consent data capture.

Dr. Gillian Hunt and Prof. Lynn Morris of the Lynn Morris Laboratory, NICD, Johannesburg, for provision of the DU179 culture supernatant, use of their RNA extraction facilities and thermocyclers.

Prof. Maria Papathanasopoulos, HIV Pathogenesis Laboratory, Department of Molecular Medicine, Wits, Johannesburg, for the donation of integrase DNA clones FV20 and FV23.

Prof. Manu Platt, Platt Laboratory, Georgia University of Technology, Atlanta, Georgia, USA, for the donation of Serum Separator Tubes.

Prof. Lee Berrie, for insight into routine viral load testing carried out at the NICD, NHLS Headquarters, Johannesburg.

Dr. Pascal Willem for use of the Nanodrop™ and RNA extraction facilities at the Department of Cytogenetics, NHLS, Johannesburg.

Inqaba Biotechnologies for the provision of Lambda phage DNA.

Poliomyelitis Research Foundation for provision of a research grant.

Faculty Research Committee Medical Research Endowment Fund for provision of a research grant.

National Research Foundation for provision of a scarce skills scholarship.

NIH PEPFAR Grant for Novel Ideas for provision of research funding and personal funding.

Wits Health Consortium, Health Economics and Epidemiology Research Office for provision of personal and travel funding.

Faculty of Health Sciences Postgraduate Research Office for assistance with travel funding.

Mr. Ramajwe Tseki, Department of Internal Medicine for timely assistance and processing of procurement of research consumables and equipment.

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Chapter 1

1.1. Introduction

Resource-limited regions of the world are often beset with a lack of basic health care resources, as effective health providers require sustained and maintained infrastructure, skilled individuals and means to communicate with the communities they serve. It is, therefore, understandable that the regions of the globe most affected by the HIV-1 pandemic are indeed resource-limited. In this regard, sub-Saharan Africa, located in the developing world, is host to the largest population of HIV-1 infected individuals, comprising 69% of the globe's new HIV infections and 70% of the world's AIDS-related deaths (UNAIDS, 2012).

Although the figures remain staggering, a 25% decrease has been recorded in new sub-Saharan infections since 2001 (UNAIDS, 2012). The main elements attributing to this success have been improved access to anti-retroviral therapy (ART). This has certainly been an important approach, since individuals with higher viral loads are at increased risk of transmitting the virus. Moreover, infected individuals who display positive treatment outcomes, through viral load suppression, lower their risk of transmitting the virus (Montaner et al., 2010). This is further displayed at the population level as sero-negative individuals inhabiting regions characterised by good ART-coverage are significantly less likely to contract the disease (Montaner et al., 2010; Tanser et al., 2013). Improved ART has also reduced the number of AIDS related deaths in South Africa (Walensky et al., 2008; Herbst et al., 2009).

The HIV-disease prognosis of a treated individual is subject to multiple factors, such as the risk of development of resistance to treatment (Deeks, 2003) and the risk of treatment interruption due to lack of adherence on part of the patient (Nischal, Khopkar and Saple, 2005), or problems surrounding provision of the drugs. The individual response to ART is highly unpredictable and therefore needs frequent monitoring. It thus follows that disease progression-monitoring plays an important role in combating the HIV pandemic. The main events occurring within the HIV disease progression timeline are: initiation of therapy, monitoring of virologic suppression, identifying virologic failure leading to ART regimen switching, identifying virologic failure leading to development of full blown AIDS and/or mortality. As long as the continued high financial and skills requirements of the current disease progression-monitoring methodologies prevent individuals in resource-limited

regions from being identified for entry into a treatment program (Stevens and Marshall, 2010; WHO, UNICEF and UNAIDS, 2010), prevention of transmission and mortality is impeded.

1.2. Literature Review

1.2.1. Methods of disease progression-monitoring

Across the spectrum of human diseases, diagnostic tests and follow-up methodologies are based on identification of symptoms and clinical signs of adverse health, as well as measurement of biomarkers (Mayeux, 2004; Jain, 2010). The observation of symptoms, while a common starting point for diagnosis, requires further confirmatory tests. This is because symptoms are often synonymous between varieties of diseases. Biomarkers are, therefore, required to identify the specific disease and causal agent. Often specific to stages of a disease, they have a primary role in disease progression-monitoring (Mayeux, 2004; Jain, 2010; Mamas et al., 2011). Here, a biomarker is by definition a biological signature associated with disease, often occurring at the molecular level (Mayeux, 2004; Jain, 2010; Mamas et al., 2011). Common biomarkers for disease include antibodies (Ballew et al., 2013), the presence or absence of intercellular signalling molecules (Lane et al., 2007), hormones (Linkov, Yurkovetsky and Lokshin, 2009), and changes in levels of molecules or cells (Mellors et al., 1997) (Jain, 2010). The identification of a biomarker involves identifying a significant difference in the particular marker when comparing a diseased and a healthy individual.

Global and national conventions recognise 2 main biomarkers used to monitor HIV disease progression: the CD4 cell count and the HIV-1 viral load. These biomarkers are implemented with specific guidelines for their use, whereby a CD4 count below 350 cells/ml is used to identify individuals for ART initiation (WHO, UNICEF and UNAIDS, 2010; Department of Health, 2013). Following treatment initiation, viral load is then recommended to identify response to treatment, specifically by identifying a change from undetectable (below 400 copies/ml) to above 1000 copies HIV-1 RNA/ml plasma (Department of Health, 2013).

The CD4 count is determined by measuring the number of cell-bound CD4 receptors in a fixed volume of blood using fluorescence activated cell sorting (FACS) (Zijenah et al., 2006). This is because cells bearing the CD4 receptor are rapidly depleted following HIV

infection (Fauci, 1988). Plasma viral load is a measure of circulating virus particles in a fixed volume of blood plasma, and specifically quantifies viral RNA stored inside the virus protein coat of infective viral particles. Because RNA degrades very easily, it is unlikely that RNA not shielded by a protein coat will be viable or present in viral load tests, which means the quantified RNA specifically represents circulating viral particles (Mellors et al., 1997), and at a ratio of 1 copy of viral RNA per particle. Viral particles circulating in the blood represent a population of viruses available to further infect and so deplete CD4 cells. It is expected therefore to be a reliable predictor of transmission risk and progression to AIDS. Although extracellular RNA is measured, the circulating population also provides information about the host's intracellular response to treatment. This is because viral particles cannot replicate outside the host's cells. If the number of circulating viral particles is increasing, this means there is active viral replication occurring intracellularly and as such would be indicative of ART failure. CD4 cell count and HIV viral load complement each other via an inverse relationship (Mellors et al., 1997), as there is an indirect correlation between the amount of replicating virus in the blood and the CD4 count. When viral replication is supressed, such as by the action of ART, the host is able to regenerate the CD4 cell supply. Should the virus begin rapidly replicating again, known as virologic failure, the CD4 cells will once again become depleted.

This inverse relationship is not however direct, as each marker differs in viability for disease progression-monitoring. The strength of the CD4 count is in its value to predict progression to AIDS disease (Korenromp et al., 2009) and is therefore the main criteria used to identify patients eligible to start ART. Still, CD4 count is not affected exclusively by HIV infection, as any state of disease can lower the count at a given moment, as well as emotional stress (Remor et al., 2007), pregnancy (Chama et al., 2009), smoking (Tollerud et al., 1989) and treatment such as cancer chemotherapy (Lissoni et al., 2009; Gunturu et al., 2010).

Fluctuations in CD4 cell count have been observed in situations of both viral suppression and treatment failure; Rawizza et al., 2011) and therefore CD4 count is not a reliable predictor of virologic failure (Badri, Lawn and Wood, 2008). The reduction in CD4 cells in response to treatment failure is delayed (Badri et al., 2008; Keiser et al., 2009). In some cases, decreases below clinically relevant thresholds, such as below 200 or 350 cells/mm^3, are evident despite an undetectable viral load (Jourdain et al., 2013). Previously, an

undetectable viral load was defined as a viral load falling below the detection limit of the relative viral load testing platform, commonly between 40-400 copies HIV RNA per ml plasma (Abravaya et al., 2003; Schumacher et al., 2007). This has been updated to below 1000 copies, which is a more standardised threshold, based on what is considered to have clinical relevance to disease progression (Department of Health, 2013). There is a level of discordance between CD4 count and viral load (Korenromp et al., 2009), which is problematic when treated individuals are managed solely by CD4 count. This is because the clinical relevance of a change in CD4 count varies depending on viral load response, which, if indicative of virologic failure, will translate into progression to AIDS or death, regardless of the CD4 count (Tan et al., 2008). Increased viral load has compared well with depleted CD4 cell count and clinical observations (Griffith et al., 1997; Mellors et al., 1997), for example, co-infection with sexually transmitted infections (STI's) (Nkengasong et al., 2001). Therefore it follows that viral load is a stronger disease progression marker than CD4 count (Mellors et al., 1997; Jourdain et al., 2013).

Viral load testing by RT-PCR

Platforms for RNA quantification and nucleic acid testing in general are complex. Viral load testing is mainly performed by real time reverse transcription – polymerase chain reaction (RT-PCR) assays (Rouet et al., 2008). The most common of these assays is the Roche Cobas Taqman HIV-1 and Abbott HIV Real Time assay. These assays provide absolute viral load counts as copies of RNA/ml plasma (Abravaya et al., 2003; Schumacher et al., 2007). Utilising what is known as probe hydrolysis, the fluorescent signal of a labelled nucleic acid probe is quenched when it hybridises to the RT-PCR amplicons (Abravaya et al., 2003; Schumacher et al., 2007). The fluorescence is monitored in real time and used to determine how early a sample reaches the exponential phase of amplification, which is then correlated to viral load (Abravaya et al., 2003; Schumacher et al., 2007).

Sensitive HIV RNA quantification requires that primers and probes target highly conserved viral gene regions, that is, regions of the viral genome which are essential to infectivity. The HIV replication process lacks proof-reading capacity which means mutations are generated in the viral genome at every round of replication leading to high levels of genetic heterogeneity in infected individuals, which could lead to underestimated viral load if the employed primers and probes cannot hybridise with these mutated regions

(Wojewoda et al., 2013). The Roche and Abbot assays detect targets in the integrase region of the *Pol* gene (Abravaya et al., 2003) and p24 region of the *Gag* gene (Schumacher et al., 2007) (Fig. 1.1.). These 2 regions are the most conserved regions of the HIV-1 genome following the long terminal repeat (LTR) region. Both genes are essential for virus infectivity. The integrase gene codes for the integrase enzyme which assists the integration of proviral DNA into the host genome (Craigie, 2001). The p24 region codes for the protein which forms the capsid around the genomic RNA within the viral particle (Freed, 1998). The LTR region is limited as a target as only a small portion of it is included in the RNA genome, while the rest of the LTR region is generated within the provirus following the reverse transcription phase of the HIV replication cycle (Cullen, 1991).

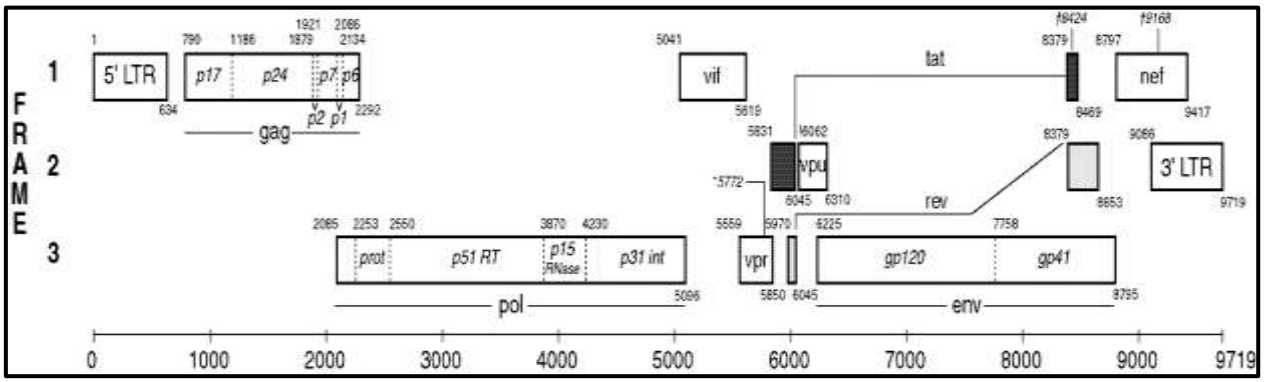


Figure 1.1 Map of the HIV-1 genome showing the p24 region of the *Gag* gene and the integrase region of the *Pol* gene, classic targets of nucleic acid-based viral load testing (Los Alamos Database, 2012).

Both assays boast excellent performance and sensitivity in terms of copy number detection limit and positive predictive value (Khopkar et al., 2013; Wojewoda et al., 2013). The Abbott assay has the highest sensitivity of the two with 100% sensitivity and the Cobas assay follows at 95%, both at a detection limit of 40 copies (Abravaya et al., 2003; Schumacher et al., 2007). They are also very specific, failing to detect common co-infections such as HBV (Khopkar et al., 2013; Xu et al., 2008), but a number of practical drawbacks make them unsuitable to resource limited settings. Assay workflow includes a variety of steps totalling 5-6 hours (Klein et al., 2003). Massive automated nucleic acid extraction devices and thermocyclers with real-time product detection are used, making the technique very expensive, difficult to operate, requiring regular maintenance and calibration. The delicate reagents, such as fluorescently-labelled probes and internal controls, require cold-storage, further increasing cost and complexity (Promso et al., 2006; Rouet et al., 2008).

Viral load testing is performed at centralised laboratories or hospitals, often situated days away from primary health care clinics (Rouet et al., 2008; Fatti, Grimwood and Bock, 2010). Primary care clinics aim to provide health care within close proximity to where people reside. However, they consist of very limited infrastructure and are staffed by a few nurses (Boulle et al., 2010) who perform both medical and administrative tasks (Ganesan-Moothusamy and Naidoo, 2013). In some settings, viral load testing does not take place at all (Boyles et al., 2011; Ganesan-Moothusamy and Naidoo, 2013). Instead CD4 counts carried out off site, combined with WHO clinical staging are used to initiate patients for ART (Ganesan-Moothusamy and Naidoo, 2013). However, WHO recommendations are less sensitive than HIV-1 RNA viral load for identifying individuals eligible for treatment and those undergoing treatment failure (Kamya et al., 2004; Sen et al., 2011). Some patients do not receive a result and a significant proportion of them are lost to follow up (Ganesan-Moothusamy and Naidoo, 2013). A loss-to-follow-up patient is a patient who enters the treatment program but does not return to the clinic thereafter (Boulle et al., 2010; Fatti et al., 2010). Since CD4 count is not as accurate as viral load, the majority of sub-Saharan HIV-infected individuals are receiving sub-optimal disease progression-monitoring (Mee et al., 2008).

1.2.2. Strategies for low-cost monitoring

The need for low-cost, simple alternatives is urgent. There are generally 2 approaches to identifying progression-monitoring strategies suitable to resource-limited settings: 1) identifying new biomarkers and 2) developing new techniques for measuring current biomarkers.

1.2.2.1. Alternative biomarkers

Disease progression-monitoring is multifaceted, including identifying the need for treatment initiation, predicting and tracking response to treatment, and predicting mortality at a clinical sensitivity and diagnostic specificity of 80-100%. Clinical sensitivity indicates how many individuals positive for any of the above disease states were identified by the relative marker, while diagnostic specificity indicates how many individuals identified as positive were truly positive (Bustin et al., 2009). A highly relevant marker would contribute to all aspects of progression-monitoring, whereas other sub-optimal markers may only be useful for monitoring isolated disease stages (Jain, 2010). Commonly investigated surrogate markers include total lymphocyte count (Mahajan, Hogan and

Snyder, 2004), β-2 microglobulin (Nkengasong et al., 2001), blood haemoglobin (Hb) (Jerene et al., 2006) and body mass index (BMI) (Maas et al., 1998) (Fig. 1.2). Further markers can include presence of co-infections (Nkengasong et al., 2001) and adherence to treatment (Cambiano et al., 2010). The merits of each of these markers are discussed below.

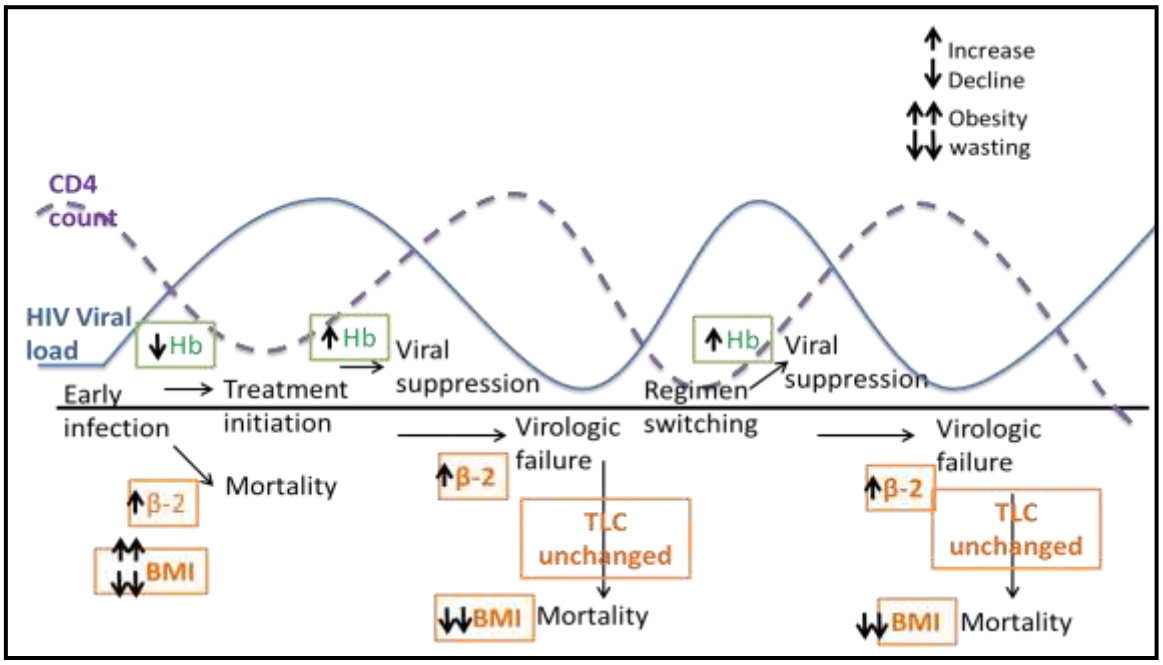


Figure 1.2 The role of four alternative biomarkers in HIV disease progression-monitoring. Biomarkers include haemoglobin levels (Hb), body mass index (BMI), total lymphocyte count (TLC) and β-2 microglobulin (β-2). Suitability of biomarkers to resource-limited settings were defined as highly relevant (green) or sub-optimal (orange).

A) Full blood count and total lymphocyte count

Full blood count (FBC) is a low-cost routine blood test from which the total lymphocyte count is easily derived. A full blood count will provide the total white blood cell count, from there the total lymphocyte count is calculated as percentage lymphocytes multiplied by the white blood cell count, multiplied by 1000 (MediaLab Inc.). It is also a logical marker as the total lymphocyte count (TLC) inherently includes the CD4 count. The simplicity of measuring TLC makes it a promising surrogate marker. The reported sensitivity for using absolute TLC to predict clinically significant CD4 count thresholds of 200 and 350 cells/mm^3 are as low as 71% and 60% respectively (Chaudhary et al., 2008). A better approach is to monitor changes in TLC, instead of using absolute values, to predict CD4 count changes which has a sensitivity of 80-90% (Florence et al., 2004; Mahajan et al., 2004; Chaudhary et al., 2008) with exception to CD4 count below 200 cells/mm^3 (Spacek et al., 2003). The clinical significance of this is that a lack of increase in

TLC following more than a month of treatment predicts death, although it is not sensitive enough to identify patients at risk of immediate death (Jerene et al., 2006). Even so, TLC does not correlate as well with viral load (Mahajan et al., 2004) and this may be due to the existing discrepancy between CD4 count and viral load. TLC also varies from individual to individual, further lowering sensitivity as a surrogate marker in isolation (Mahajan et al., 2004). The prognostic value of TLC is therefore improved when combined with the individuals' WHO stage (Jerene et al., 2006; Chaudhary et al., 2008) or with white blood cell count and CD4 count at treatment initiation (Azzoni et al., 2012). Although the main advantage of this test is the minimal cost, specimens are nevertheless still sent to reference laboratories for result interpretation (Ganesan-Moothusamy and Naidoo, 2013), increasing turn-around-time.

B) β-2 Microglobulin and immune activation

Immune activation refers to the rapid increase in circulating lymphocytes and cytokines in response to infection (Cossarizza et al., 2012). A positive feedback exists in HIV infection whereby an increase in viral load, interpreted as increased infection leads to immune activation, following that immune activation aids HIV replication due to the activation of CD4 cells (Cossarizza et al., 2012). It can therefore be assumed that markers of immune activation are multi-functional in HIV disease progression-monitoring by identifying increased viral load which points to treatment failure while simultaneously predicting disease progression due to CD4 depletion following increase viral replication. β-2 microglobulin is a marker of immune activation for which increased levels are associated with increased viral load (Nkengasong et al., 2001; Cossarizza et al., 2012). A range of 2.9-5 mg/L predicts death in ART-naïve patients (Nyamweya et al., 2012). Measurement can be performed simply by nephelometry (Savès et al., 2001). A nephelometer measures the scatter of light in liquid through which antigens and antibodies are passed, allowing for the detection of antibody-antigen immune-complexes. Even though β-2 microglobulin levels do change in response to ART, the correlation is still below 90% clinical sensitivity and specificity for predicting virologic failure, as well as with predicting disease progression (Chaudhary et al., 2008), which compares sub-optimally with the statistically significant correlation between HIV-1 RNA viral load and these 2 disease stages (Mellors et al., 1997). Another means of identifying immune activation includes monitoring the presence of acute co-infections, such as symptoms of STI's and/or the presence of inflammation (Nkengasong et al., 2001), although such a strategy may be a more

qualitative than a quantitative means of monitoring disease. The advantage of immune activation is that it is a very accurate means of predicting increased viral load, however markers of immune activation such as β-2 microglobulin lack sensitivity (Mellors et al., 1997) since an ideal clinical sensitivity should be above 90%.

C) Haemoglobin (Hb)

The investigation into Hb as an HIV disease progression marker arose from the high incidence of anaemia, defined as Hb less than 12 g/dL (Obirikorang and Yeboah, 2009), observed in HIV positive individuals (Semba et al., 2002). Hb levels are tested on a rapid, finger-prick, point of care device and are already performed frequently at primary health care clinics. While levels correlate significantly well with CD4 count, dropping below healthy ranges (12.0-18.0 g/dL) with the clinically relevant decline in CD4 count (Semba et al., 2002; Obirikorang and Yeboah, 2009), this only translates into a clinical sensitivity of around 50% in identifying individuals for ART initiation (Sen et al., 2011). Decline in Hb in pre-ART anaemic individuals predicts a negative prognosis of progressing to AIDS (Jerene et al., 2006), while individuals who develop anaemia during the course of HIV infection have a mortality rate of 37%, in both pre-ART non-anaemic and anaemic individuals (Semba et al., 2002). Following successful ART treatment, individuals anaemic at treatment initiation exhibit significant recovery from anaemic levels of Hb (Johannessen et al., 2011), showing that routine Hb testing can also play a role in monitoring treatment response.

D) Body mass index (BMI) and obesity

Body mass index (BMI calculated as weight divided by height squared, measured in kg/m^2) is by far the simplest biological characteristic to monitor. The clinical relevance of a decline or increase in BMI depends on identifying a baseline BMI (van der Sande et al., 2004), that is the BMI of an individual at the start of treatment. This is because weight loss can be considered pathological if it equates to a BMI less than 18 kg/m^2 (Malvy et al., 2001; van der Sande et al., 2004) or is more than 10% unintentional weight loss (Maas et al., 1998) or can be indicative of a healthy lifestyle change for a previously obese individual. Obesity at baseline is associated with faster progression to disease, contributing to CD4 cell depletion (Crum-Cianflone et al., 2010). Alternatively, a decline in a treated patient with a low baseline BMI suggests poor response to treatment (Jerene et al., 2006) this does not specifically correlate to viral load (Maas et al., 1998). Prior to improved

longevity as a result of improved access to treatment, also known as the pre-ART era, low BMI, indicative of wasting, has been a strong predictor of progression to AIDS (Maas et al., 1998; Malvy et al., 2001). Wasting is considered generally to be a good predictor of all-cause mortality and is also useful as an ART-eligibility criterion (Maas et al., 1998; van der Sande et al., 2004).

E) Clinical presentation and co-infections

The most obvious predictor of disease progression is the presentation of disease as it points to significant immune-compromise, which is suggestive of progression to AIDS. WHO and the department of health include the presence of co-infection with either TB or Hepatitis B as part of the guidelines for identifying individuals eligible for ART (WHO, UNICEF and UNAIDS, 2010; Department of Health, 2013). Since increased viral load is associated with disease progression, it is unsurprising that the presence of multiple STI's is associated with increased viral load, as high as 2.5 fold (Nkengasong et al., 2001). Such infections fortunately have very distinct clinical presentations that aid immediate diagnosis and can therefore be easily included in a resource-limited monitoring strategy. Other co-infections common to the HIV-positive population such as Hepatitis B and C (HBV, HCV) and TB show potential to predict mortality and point to immune suppression (Chen et al., 2009; Glynn et al., 2010). However, clinical presentation of advanced co-infection induced disease, such as active T.B. is an indication of advanced progression to AIDS (Ong et al., 2008), which is too late a stage to predict virologic failure or disease progression, possibly due to discordant or delayed CD4 response to virologic failure. Still, presence of advanced disease is a very important indicator of risk of mortality.

F) Adherence to treatment

Standard ART consists of the combination of at least three antiretroviral drugs. ART regimens administered in South Africa consist of a variety of anti-retroviral drugs including Nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside Reverse Transcriptase inhibitors (NNRTI) (Department of Health, 2013). The required daily consumption of multiple pills along with reported adverse side-effects such as nausea and appetite loss make adherence to treatment a challenge. Treatment interruption can proceed to treatment failure due to the propagation of resistant viral strains during treatment lapse. Therefore, monitoring patient adherence can be used to monitor disease progression (Townsend et al., 2007; Bisson et al., 2008; Cambiano et al., 2010). Virologic failure can

be predicted at a sensitivity of 80% by identifying patients who adhered to less than 90%-100% of their treatment over a 6 month period or 90% over a 12 month period of monitoring adherence (Bisson et al., 2008). The advantage of the use of adherence monitoring is it predicts virologic failure at a specificity of 88-98% (Bisson et al., 2008) and is performed simply by ART prescription data capture, but the major drawback is that, when applied in a diagnostic context, the turn-around-time is at least 6 months and therefore cannot identify treatment failure immediately.

Diagnostic accuracy of markers

Measurements for most of these markers are easily obtainable at the point of care. Each marker shows statistically significant correlations (direct or inverse) with CD4 count and/or viral load (Florence et al., 2004). But when these correlations are applied in a diagnostic context both sensitivity and specificity is lacking. Apart from Hb, they fail to approach the prognostic superiority of the CD4 count and HIV viral load. Even so, in the absence of CD4 count and viral load measurements, the value of these markers in assessing HIV disease progression is strengthened when combined. BMI for example, affects Hb levels (Semba et al., 2002) and therefore one could expect that a decline in Hb will have different implications when comparing individuals with healthy and unhealthily low BMI's. Similarly, the use of Hb measurements aids in TLC prediction of CD4 count below 200 cells/mm^3 (Spacek et al., 2003). Such markers may therefore be applied algorithmically as a low cost monitoring strategy (Azzoni et al., 2012).

1.2.2.2. Alternative viral load assays and point-of-care strategies

Given the superiority of HIV-1 viral load in HIV disease progression-monitoring, another approach to improving monitoring in resource-limited settings is the development of simpler, cheaper and faster methodologies of viral load measurement. The long terminal repeat (LTR) assay (Drosten et al., 2006), reverse transcriptase activity assay (Jennings et al., 2005) and p24 ELISA (Schupbach et al., 1996) are examples of cheaper viral load assays which have already been developed. Pooled viral loads (Smith et al., 2009), and the use of dried-blood-spots (Johannessen et al., 2009), are developing technologies that aim to ease the challenge of centralised real time RT-PCR testing. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) on the other hand is an example of a novel nucleic acid technology that may be comparable to real-time RT-PCR nucleic acid test (Notomi et al., 2000).

A) LTR assay

This assay is a one-step real time RT-PCR assay. While this assay compared well with the commercial kits, it still relies on the use of the real-time thermocycler (Fibriani et al., 2012). While simultaneous cDNA synthesis and amplification reduces the cost of reagents, complexity and turn-around-time (Drosten et al., 2006), there is no simple read out, reagents include multiple probes and an internal control (Fibriani et al., 2012) rendering this assay unsuitable for remote regions. It may be considered a good alternative option in the context of centralised laboratories as it appears to be superior in multiple subtype detection, cheaper than real-time PCR and also ultrasensitive, with a detection limit of 50 copies/ml (Drosten et al., 2006) and over 90% sensitivity and specificity for identifying treatment failure as a HIV-1 RNA viral load above 500 copies/ml (Fibriani et al., 2012).

B) RT activity assay

Reverse transcriptase (RT) activity assays aim to quantify the amount of circulating virus by measuring the activity of the virus' inherent RT (Jennings et al., 2005; Stevens et al., 2005; Mine et al., 2009). The assay involves immobilizing RT and quantifying DNA hybrid products using labelled deoxy-uridine tri-phosphates (dUTPs) (Jennings et al., 2005; Stevens et al., 2005; Mine et al., 2009). Performance and result interpretation of such an assay is somewhat complex; and the 2-day turn-around-time is highly unfavourable (Iqbal et al., 2007) and contributes to high rates of loss as patients do not return to the clinic for their results.

C) p24 Enzyme-linked immunosorbent assay (ELISA)

The p24 enzyme-linked immunosorbent assay (ELISA), which quantifies viral p24 antigen in plasma (Schupbach et al., 1996; Stevens et al., 2005; Patton et al., 2006), is simple, and comparable to the current PCR tests, with a 100% sensitivity for samples of above 50 000 HIV RNA copies/ml (Jennings et al., 2005). It is not limited by genetic diversity, which would be a limitation in a PCR-based test, since it measures the actual protein levels (Patton et al., 2006). The assays can be modified to accommodate use of dried blood spots (DBS) as well as fluid (Patton et al., 2006). However, despite inexpensive reagents, which provide a cost advantage, the regular assay takes approximately 2 days and requires a reasonably skilled laboratory technician to monitor multiple incubation steps and quantification by spectrometry (Patton et al., 2006). Also, the lower detection limit of 50

000 copies HIV is 50 times higher than the clinically relevant viral load threshold defining virologic failure.

D) Pooled viral load testing

Pooled viral load testing is a cost-reducing strategy for resource-limited viral load monitoring, involving performing a single viral load test on pooled patient samples of a particular infected population. It is performed most effectively, if the tested population has a predicted low risk of virologic failure (van Zyl et al., 2011). This is an example of how a combination of the prior discussed clinical biomarkers can complement RNA viral load testing. The pooling is done in 2 ways, either by pooling 5 samples or by a matrix with the capacity to pool 100 samples, where a single sample may be pooled more than once (Smith et al., 2009; van Zyl et al., 2011). Within the pools, individual samples are also tested to help identify which samples contribute most to the viral load total measured for the pool, thereby identifying individuals for treatment failure without having to test those who are likely to have a low viral load (Smith et al., 2009; van Zyl et al., 2011). In this manner costs are reduced, as the number of viral load assays performed can be reduced by 70% without compromising the negative predictive value for individuals samples, reported to be 80-100% respective of increasing viral load threshold (Smith et al., 2009). Pooled viral loads, therefore, offer a solution to expense aspects of the disease progression-monitoring challenges.

E) Dried blood spots (DBS)

Conventional viral load tests are performed on plasma, the collection of which requires skilled phlebotomists and a means of isolating the plasma from whole blood. Dried blood spot (DBS) technology is a point-of-care method of sample collection that simply involves spotting a small volume of blood, possibly a finger-prick, onto filter paper and allowing to dry overnight, before sending to a centralised routine viral load laboratory for RNA extraction and subsequent testing (McNulty et al., 2007; Neogi et al., 2012). DBS use displayed clinical sensitivity and diagnostic specificity of 100% for predicting plasma HIV-1 viral load of above 1000 copies/ml (Neogi et al., 2012), that reduces with reduction in viraemia (Johannessen et al., 2009). Combination with Nucleic Acid Sequence Based Amplification (NASBA) viral load testing resulted in 91% and 97% sensitivity and specificity respectively for predicting virologic failure (Johannessen et al., 2009). Although dry-blood spots also contain proviral DNA (McNulty et al., 2007), this does not affect the

quantified result if DBS-modified nucleic acid testing platforms are used, such as NASBA, which only amplifies RNA (Johannessen et al., 2009). The advantage of DBS technology is it minimises blood sample processing and volume of blood handled by primary healthcare workers (McNulty et al., 2007; Johannessen et al., 2009; Neogi et al., 2012) making it a safer, simpler and cheaper alternative to venepuncture.

Both the p24 and RT assays are still a challenge in resource-limited settings due to the skills required for their complex operation. Point of care platforms and technologies improve access to disease progression-monitoring and timely initiation of treatment and regimen switching. Still, a number of challenges affect the efficacy of such technologies in the resource-limited settings. Lack of stringent adherence to operation instructions can generate a high rate of false-negative results (Wolpaw et al., 2010). The importance of adhering to the specified time-to-result readout are of particular concern (Wolpaw et al., 2010), and the importance of this should be well demonstrated as part of training. Point-of care technologies are also limited by lower sample capacity than the current gold standard tests, and the use of miniaturised sample input volumes can compromise accuracy (Wu and Zaman, 2012). The cost per test of point-of-care platforms is generally lower than the equivalent gold-standard, permitting short-term cost savings. But often costs of continued use, including requirements for additional reagents or biological waste disposal are overlooked, which may in the long-term equate to costs of the current centralised platforms (Wu and Zaman, 2012). This cost is further expounded when considering that these devices often focus on just 1 aspect of disease monitoring (Wu and Zaman, 2012; Myer et al., 2013) and multiple devices and consumables or reagents may be required to be stocked at primary health-care clinics to diagnose and monitor several conditions (e.g. HIV and TB) and various stages of the disease process. Personnel running these tests will need sufficient training for all of these devices.

F) Loop Mediated Isothermal Amplification (LAMP)

LAMP is a novel molecular technique which mediates the amplification of nucleic acids isothermally. The LAMP reaction is a favourable option for diagnostic assays due to the low cost of the technique. The only equipment required to perform the reaction is a heating block or 2, a bench-top centrifuge and pipettes to add the nucleic acid to the LAMP reaction mixture. Most targets can be detected within 45 minutes to an hour incubation period (Zhang et al., 2010). Due to the ‘all-in-one’ capacity of the reaction, many LAMP

assays are being developed for detection of a variety of pathogens including HBV, Rift valley fever virus (Le Roux et al., 2009), TB (Boehme et al., 2007), HCV (Reddy et al., 2010), H1N1 influenza (Ma et al., 2010) and HIV-1 (Curtis et al., 2008; 2009), using both DNA and RNA (RT-LAMP) (Soliman and El-Matbouli, 2009; Buates et al., 2010) as template. These assays are simple to perform and interpret, making them even more advantageous for resource-limited settings (Boehme et al., 2007). While quantitative assays are also being developed (Cai et al., 2008) this has not yet been done for HIV-1 and the technique is yet to be implemented commercially.

1.2.3. LAMP mechanism

Similar to PCR, the LAMP reaction takes place by oligonucleotide-primer directed addition of deoxyribo-nucleotides to a growing chain of DNA, catalysed using a high-strand displacement activity polymerase (Notomi et al., 2000). In conventional PCR, heat denaturation, between 95°C - 97°C, brings about template and daughter strand displacement. This serves to initiate each cycle of PCR, after which oligonucleotide primers can bind to the now single-stranded DNA. This step is not required in the LAMP reaction as the *BSt* Polymerase catalysing the reaction displays characteristically high strand displacement activity, which is further mediated by the interaction between 2 distinct pairs of primers, namely outer primers (F3 and B3) and loop-generating inner primers (FIP and BIP) (Notomi et al., 2000). The inner primers consist of 2 sequences, 1 complimentary to the target and 1 which is identical to a sequence further downstream, forming a 5' overhang when the first piece binds (Fig. 1.3) (Notomi et al., 2000). The overhang will subsequently loop back and bind to its complement on the daughter strand (Fig. 1.3) (Notomi et al., 2000). As this happens, outer primers, which are much like the forward and reverse primers used in PCR, will bind upstream to the inner primers, displacing the stem-loop structured, daughter strand (Fig. 1.3) (Notomi et al., 2000). Therefore, with no need for heat denaturation at the start of each reaction, LAMP is run at a temperature equivalent to the annealing step of PCR and terminated at 85°C within an average incubation period of 1 hour.

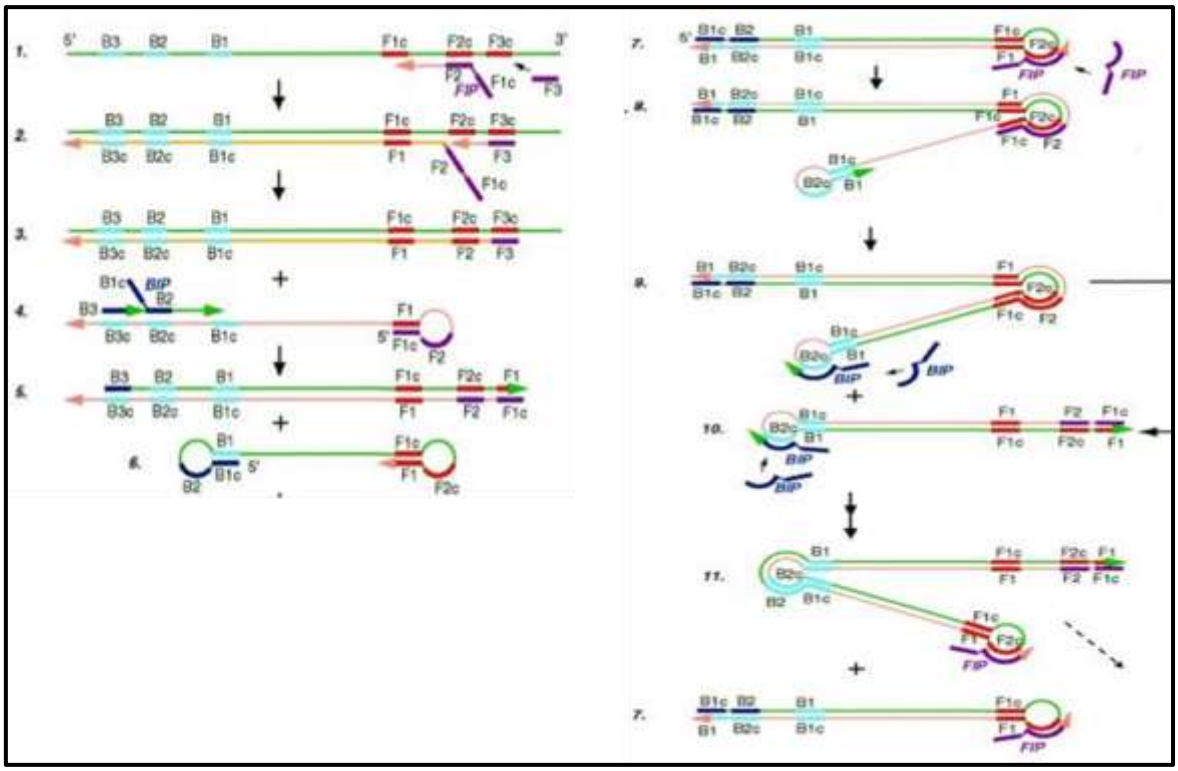


Figure 1.3 Schematic diagram of the mechanism of loop-mediated isothermal amplification (Notomi et al., 2000). 1) Initial binding of FIP and F3 primers to the template nucleic acid; 2) Strand displacement is achieved by displacement of the partially single-stranded, extended FIP primer by extension of the upstream F3 primer hybridised to the same strand; 3) The double-stranded DNA product following extension of primer F3; 4) The F1c portion of the FIP strand loops back due to extension of FIP passed the complement of F1c (F1), to which BIP and B3 is able to hybridise and begin a chain reaction; 5) Extension of the BIP primer causes the unravelling of the loop formed by FIP in step 4 to form a double-stranded amplification product; 6) Subsequent amplification products can consist of loops formed both by FIP and BIP; 7) The single-stranded nature of the loops in the amplification products can hybridise to free FIP (or BIP) to permit autocycling; 8-10) Continous formation and unravelling of loops as a result of continued primer-hybridisation and primer extension-induced strand displacement permits amplification of DNA with varying multiples of stem-loop regions.

A number of adaptations and applications to LAMP have emerged since its initial development by Notomi et al. (2000). A pair of loop primers additional to the original 2 outer and inner primers was added (Nagamine, Hase and Notomi, 2002). These loop primers hybridise to the loops of the LAMP amplicons just between the F1 and F2 regions, and will therefore only bind to loops formed from the target sequence (Nagamine et al., 2002). The main function of these extra primers is to speed up the auto-cycling phase of the reaction (Nagamine et al., 2002).

Apart from DNA, LAMP has been adapted for RNA amplification with the addition of a robust reverse transcriptase (RT) with the LAMP reaction mixture, allowing for simultaneous cDNA synthesis and amplification in a single step (Notomi et al., 2000;

Buates et al., 2010; Cardoso et al., 2010). The activity of *AMV* RT, for example, is optimal at temperatures between 50°C and 65°C, which fits in with the LAMP incubation temperature range required by *BSt* Polymerase.

A) Analysis of LAMP amplification

Magnesium pyrophosphate ($Mg_2P_2O_7$) produced as a phosphate group is released with dNTP incorporation into the growing DNA chain, which reacts with magnesium sulphate in the LAMP reaction. As amplification continues, the by-product builds up in a manner which changes the turbidity of the reaction mixture (Mori et al., 2004; Yoshikawa et al., 2004). As a result, a turbidometer can be coupled to an isothermal heating device to monitor by-product accumulation in real time (Mori et al., 2001; Le Roux et al., 2009). Through this, Mori et al. (2001) established a correlation between the increase in $Mg_2P_2O_7$-induced turbidity and accumulation of LAMP-amplified DNA. This suggests that a means able to detect the amount of by-product should indirectly reflect the amount of DNA produced.

The by-product forms a white precipitate following pulse-centrifugation of the inactivated reaction mixture (Le Roux et al., 2009). $Mg_2P_2O_7$ can further be detected using metal-chelating dyes such as hydroxy-naphthol blue (HNB) (Zippelius et al., 2000; Goto et al., 2009; Ma et al., 2010; Hadersdorfer et al. 2011). HNB behaves stably in the LAMP reaction mixture, changing it from violet to blue upon interaction with the free magnesium ions (Zippelius et al., 2000; Goto et al., 2009; Ma et al., 2010; Hadersdorfer et al. 2011).. It has been employed for end-point detection in the LAMP reaction to determine a qualitative positive or negative result, as with H1N1 influenza for example (Ma et al., 2010) but this has not been applied in the context of HIV. The sensitivity of HNB is comparable to dyes that directly detect DNA products, detecting the same number of amplification-positive reactions as SYBR Green (Goto et al., 2009). It does not interfere with amplification and therefore can be added to the reaction mixture prior to incubation (Goto et al., 2009). This limits opportunities for incorrect result interpretation via cross contamination following opening the tube post-amplification. Calcein, similar to HNB, might also be used for LAMP detection (Liang et al., 2009) however, it increases reaction cost, as it is a fluorescent dye that requires a UV light source for result interpretation adding to equipment costs and the requirement for further skills or training (Goto et al., 2009). It is 10 times less sensitive than HNB compared to SYBR Green (Goto et al., 2009).

Other more specific detection methods, also used for quantitative PCR have been employed, such as addition of SYBR green (Reddy et al., 2010; Zhang et al., 2010), Gel Red™ (Nakao et al., 2010) or fluorescent probes (Curtis et al., 2009). SYBR green fluoresces when intercalated with double-stranded DNA for which the emitted fluorescence can be measured and compared against a set of quantitative standards (Skeidsvoll and Ueland, 1995). Probes on the other hand are single-stranded sequences of DNA, similar to oligonucleotide primers, attached to a fluorescent tag (Abravaya et al., 2003). Although the specificity of such technologies is desirable, cost is a major drawback with regard to implementing them in a low-cost, point of care assay. SYBR green is an expensive dye, as are fluorescent probes and fluorescence-detection apparatus. Further, fluorescent materials are photodegradable with stringent storage requirements. Such technologies also require added controls against false positive results, pertaining to background fluorescence or malfunction of apparatus, so adding to the complexity of such a test.

B) LAMP specificity and sensitivity

The LAMP reaction is very specific as the requirement for 4 to 6 primers targeting 6 to 8 regions of the same gene makes it nearly impossible to correctly amplify non-template nucleic acids (Notomi et al., 2000). Assays developed for detection of viral pathogens have proved to be strain-specific (Yoshikawa et al., 2004), since the same virus infecting a species different to the target host could not be detected. This is important for HIV viral load testing in South Africa, as of the 2 types of HIV, HIV-1 and HIV-2, HIV-1 is most prevalent; specifically sub-type C (van Harmelen et al., 1999). It is suggested that efficiency of amplification is hindered when using primers designed to regions with lower than 90% sequence identity (Zhang et al., 2010).

Sensitivity of LAMP varies from application to application, with some studies reporting amplification of as little as 10 copies of the target (Nakao et al., 2010; Reddy et al., 2010) or no less than 200 copies (Cai et al., 2008) depending on the detection format (Yoshikawa et al., 2004). If needs be, LAMP sensitivity can be adjusted by adjusting the reaction time (Yoshikawa et al., 2004; Hadersdorfer et al., 2011). A low sensitivity can be exploited for threshold copy number quantification. For example, a LAMP assay with a 100 copy detection limit will successfully distinguish between a sample containing 100 copies of the target or a sample with more copies, as the 100 copy sample will appear negative. This is

highly useful for an assay for which the clinical decision does not require an absolute value, such as that of HIV viral load, whereby virologic failure is defined as anything above 1000 copies HIV RNA/ml plasma (Department of Health, 2013).

Amongst the discussed approaches, LAMP seems to be the most promising. Although not commercialised, there is evidence that the reaction can be converted to a point of care format (Boehme et al., 2007; Bearinger et al., 2011; Njiru, 2012). Further advantages essential to the resource limited setting include a same-day turn-around-time and limited infrastructure required. Still, an RT-LAMP based viral load assay remains to be developed.

1.3 Study aims and objectives

The aim of this study was to identify or develop a low cost and simple means of assessing disease progression in HIV infected individuals, which would have the potential of being suited to a resource-limited setting.

Various clinical markers were investigated in the literature, of which none were deemed favourable, either due to the lack of sensitivity or specificity of these markers as predictors of disease progression, or due to the complexity of the techniques used to measure them.

Alternative techniques for measuring the conventional markers, specifically viral load, were also reviewed. The most promising of these techniques was a novel nucleic acid based RT-LAMP technique. The technique uses cheaper reagents than others and only requires a heating block and centrifuge to perform. The simplest diagnostic tests are analysed by colorimetric indicators visible to the naked eye, such as the conventional HIV antibody tests and urine dipstick tests. RT-LAMP can also be analysed this way when combined with the hydroxy-naphthol blue, metal-chelating dye. The kinetics of the RT-LAMP assay are similar to real-time PCR, whereby a larger amount of template is amplified faster than a smaller amount. Added to this is a limited sensitivity, amplifying no less than a 100 copies of template in 60 minutes.

Taking all of these characteristics of the technique into consideration, the RT-LAMP technique, combined with the colorimetric HNB dye was chosen as the basis for a low-cost viral load assay. The specific aim of this investigation was to test if the RT-LAMP technique could be applied semi-quantitatively for HIV-1 viral load monitoring by exploiting the reaction between the RT-LAMP by product and HNB so that a colour change from violet to blue would be indicative of a clinically relevant detectable viral load, such as above 400 copies or 1000 copies of HIV-1 RNA per ml.

Assay development consisted of optimising HIV RNA template preparation and the various components of the RT-LAMP reaction by:

i) Evaluating various methods of isolating plasma from whole blood
ii) RT-LAMP primer design against 2 of the most conserved gene regions of HIV-1 RNA genome: Integrase and p24
iii) Determining optimal concentrations of RT-LAMP reagents for highest assay efficiency
iv) Determining the optimal conditions for stable dye interactions
v) Investigating whether HNB dye can be applied semi-quantitatively

vi) Testing the optimised RT-LAMP assay on RNA obtained from HIV-1 infected individuals undergoing routine viral load testing by real-time RT PCR.

The methods used to achieve the above objectives included:

i) Viral RNA extraction from HIV-1 positive whole blood, and 2 control samples: the WHO 2nd standard for HIV-1 (sub-type B-spiked plasma) and culture supernatant of HIV-1 sub-type C isolate DU179

ii) *In silico* primer design

iii) RT-LAMP

iv) Qualitative and quantitative agarose gel electrophoresis

v) Restriction digestion analysis

Chapter 2

2.1 Methods

RT-LAMP viral load assay development

Studies have shown that the strongest tool for monitoring progression of HIV disease is measurement of viral load (Jurriaans et al., 1994, Mellors et al., 1997). The most direct means of measuring viral load is by detecting the virus genetic material using nucleic acid based techniques. Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) is a low-cost, simple, dynamic nucleic acid amplification technique (Notomi et al., 2000). This technique was therefore selected as the basis of the low-cost, rapid viral load assay.

LAMP is a means of isothermal amplification of nucleic acid (Notomi et al., 2000). The reaction amplifies RNA when accompanied with a suitable reverse transcriptase (RT), termed RT-LAMP (Notomi et al., 2000; Buates et al., 2010; Cardoso et al., 2010). The use of RT-LAMP with the aid of hydroxy-naphthol blue dye (HNB) in a viral load detection application is a novel approach, both to HIV-1 viral load detection and RT-LAMP. The work represented below was performed at the Medical Oncology Research Laboratory at the Department of Internal Medicine, Faculty of Health Sciences. Biosafety clearance (number IB110502) was obtained for work performed on live HIV-1 virus. Ethics clearance (certificate number M10418, Appendix A) was obtained from the Human Ethics Research Committee for collection of HIV-infected and non-infected human whole blood upon informed consent.

It was necessary to first optimise the assay with the purpose of ensuring that the assay distinguishes between detectable and undetectable viral load consistently. This initial optimisation phase consisted of designing and testing the LAMP oligonucleotide primers; determining concentrations of various reaction components for optimal RT-LAMP amplification; extrapolating the minimum duration of the reaction upon which a colour change from violet to blue indicates a detectable viral load, at least 1000 copies HIV RNA/ml; and applying the optimised reaction conditions to attempt to semi-quantify viral load results of 10 South African HIV positive samples.

2.1.1 Sample preparation and RNA extraction

The RT-LAMP viral load assay was developed using 3 sources of live HIV virus being: whole blood of infected individuals, WHO 2nd international HIV-1 standard plasma (NIBSC) (Appendix B) and HIV-1 culture supernatant isolate DU179, a gift from the Lynn Morris virology lab (The Centre for HIV & Sexually Transmitted Infections, NICD, Johannesburg). Whole blood was also collected from 5 HIV-negative, healthy volunteers. Following informed consent (Certificate no. M10418, Appendix A), whole blood was collected in EDTA vacutainer tubes (Becton, Dickinson, New Jersey, USA) from HIV positive individuals attending the Themba Lethu clinic at Helen Joseph Hospital, Johannesburg, South Africa. WHO standard plasma (NIBSC, Hertfordshire, UK) and HIV-1 culture supernatant, produced off-site, were obtained for use as controlled sources of live virus. Virus culture supernatant is the viral extract obtained from infected cell-culture. Generally, lymphocytes are cultured and infected with a single, homogenous isolate of HIV-1. With the replication of the virus, progeny are released into the growth medium of the lymphocytes. The progeny are then isolated by centrifugation of the culture producing an intact, cellular pellet and supernatant consisting of homogenous suspension of progenic virions.

Conventional viral load testing is performed on RNA extracted from patient's plasma. Plasma is used because it is a cell-free component of the blood. Also, the proviral DNA and viral transcript RNA contained in the cell do not represent the number of replicating virions. Thus, by excluding infected cells, this ensures that only the genomic RNA of the virus is counted. Although plasma is cell-free, it may contain protein-material and salts which interfere with purity and quality of extracted RNA. In order to optimise the process of obtaining HIV-1 genomic RNA, several methods of plasma isolation and pre-extraction plasma preparation were explored:

A) Plasma isolation

i) Plasma was isolated from whole blood by centrifugation at 1000xg for 5 minutes. The clear plasma supernatant was transferred to cryovials (Greiner-Bio) at a total volume of up to 3 ml and stored at -20°C by slow-freezing, until required.

ii) Whole blood collected in EDTA-coated collection tubes (Becton, Dickinson, New Jersey, USA) was centrifuged at 12000xg (4000 rpm) for 20

minutes then transferred to cryovials (Greiner-Bio) at a volume of up to 3 ml and stored at -70°C, until required.

iii) Whole blood collected in EDTA-coated collection tubes (up to 5 ml) or previously isolated plasma (1 ml) was transferred to a serum separator tube (SST) (Becton, Dickinson, New Jersey, USA) and centrifuged at 1100xg for 15 minutes. Note that serum separator tubes are blood collection tubes which contain a gel substance that liquefies during centrifugation allowing cells and proteins to sink below the gel, while the serum remains above it. When centrifugation is stopped, the gel solidifies, creating a barrier between serum and blood cells. The supernatant above the gel barrier was transferred to cryovials (Greiner-Bio) in 1 ml aliquots and stored at -70°C until required.

B) Pre-extraction preparation

i) Plasma

It has been suggested that viral concentration improves RNA yield and quality. The Qiagen RNA extraction (Qiagen, Hamburg, Germany) procedure which was used requires 140-200 µl of plasma as starting material. For conventional viral load testing, the starting volume of plasma is 1 ml. Therefore, in order to keep to conventions, viral particles occurring in 1 ml of plasma were concentrated via centrifugation to a volume appropriate for use in the Qiagen extraction procedure. Concentration was performed by thawing plasma on ice, vortexing and transferring 1 ml into 1.5 ml Eppendorf tubes which were centrifuged at 23000xg for 60 minutes at 4°C. The resulting pellet was resuspended in plasma (200 µl) if obtained from plasma, or phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, USA) (200 µl) (Appendix B) if obtained from culture supernatant and kept on ice before proceeding with the Qiagen Viral RNA extraction procedure.

ii) HIV-1 isolate DU179 culture supernatant

Culture supernatant was stored at -70°C in 1 ml aliquots until required, to eliminate negative effects of repeated freeze-thaw cycles. Culture supernatant (1ml) was thawed on ice and subjected to viral particle concentration as described in (B.i) above and the pellet resuspended in PBS (200 µl) (Appendix B) before proceeding with the extraction.

C) RNA extraction

RNA was extracted from all of the above preparations using a silica-column based method specific for viral nucleic acid extraction from cell-free fluids (Qiagen). Traditionally, a phenol-chloroform method is employed using various buffers that allow isolation of the RNA from protein, polysaccharides and DNA (Blackburn and Gait, 1996). A guanidine thiocyanate (GuTc) buffer, also containing sodium salt (PrimerDigital), causes the precipitation of polysaccharides and DNA, which is pelleted following centrifugation, and the RNA-containing supernatant is then removed for further processing (Blackburn and Gait, 1996). GuTc is a chaotropic agent which assists the RNA extraction process in 2 ways, by chaotropic disruption of nuclease enzymes which destroy nucleic acid; and by disrupting the protein structures of the viral particles (Blackburn and Gait, 1996). The supernatant is next treated with phenol repeatedly, which allows for isolation of the RNA from proteins by separation into visible phases (PrimerDigital). The phenol phase contains the proteins, while aqueous phase, containing nucleic acids, can be removed from the solution and washed with chloroform-isoamyl alcohol (Blackburn and Gait, 1996). The chloroform removes residual phenol while the alcohol (ethanol is also used), precipitates the RNA.

Similarly, the Qiagen procedure consists of lysing the viral particles by incubation of the cell-free fluid in a lysis buffer containing GuTc (buffer AVL), except RNA isolation is simplified as RNA can bind to a silica-membrane under high salt conditions. Ethanol is added to the lysate to adjust the pH for nucleic acid binding to the silica column. Polysaccharides, proteins and other debris present in the lysate are pelleted into a collection tube attached to the silica column during centrifugation. Instead of requiring transfer of the supernatant, the RNA is retained with the silica column for further processing, eliminating the need for repeated phase separation. The column is then washed with 2 wash buffers (AW1 and AW2), the first containing Guanidine chloride and ethanol, repeatedly to remove residual lysate and buffer, to ensure that the extract does not contain contaminating proteins or chemicals that would compromise the quality of the RNA. The guanidine chloride in this step causes chaotropic disruption of contaminants and nucleases. In the second wash step, the ethanol concentration is changed to prepare the RNA extract for elution from the column. Lastly, the extract is eluted from the column by incubating the silica membrane with nuclease-free dH_2O or a low salt buffer, which is unfavourable for

binding to silica, and aids elution of the RNA, followed by high-speed centrifugation to release the extract from the column.

i) Qiagen Viral RNA extraction

The extraction was performed as per Qiagen's instructions. Starting material (200 µl), of either plasma or virus resuspended in PBS, was combined with buffer AVL™ (560 µl) and carrier RNA (5.6 µl) provided with the kit (Appendix B), pulse-vortexed for 15 seconds and incubated at ambient temperature for 10 minutes. RNA occurring in very small amounts is susceptible to degradation. The addition of carrier RNA increases the concentration of RNA on the column and in the eluate, providing stability for low yields of RNA. Following lysis, 100% molecular grade ethanol (560 µl) (Sigma-Aldrich, St Louis, USA) was added and pulse-vortexed for 15 seconds. Thereafter, the lysate (630 µl) was applied to the provided silica column and centrifuged at 6000xg (8000 rpm) for 1 minute. This was repeated on the same column for the remainder of the lysate, followed by washing the column of residual buffer and plasma in 2 steps with buffers AW1™ (500 µl) and AW2™ (500 µl). Before elution it is recommended to dry the column of residual buffer by an added centrifugation step at 8780xg (14000 rpm) for 1 minute. It was tested whether inclusion or exclusion of this additional step optimised yield and quality of the RNA extract. Although inclusion of the step is recommended by the manufacturer, it was found that exclusion lead to better yield and quality based on Nanodrop™ Spectrophotometry (see below). RNA was eluted by on-column incubation of nuclease-free dH_2O (30 µl) (Thermo Scientific, Massachusetts, USA) for 5 minutes at ambient temperature, before centrifugation at 6000xg (8000 rpm) for 1 minute. RNA was quantified and qualified using NanoDrop™ (Thermo Scientific, Massachusetts, USA). The Nanodrop™ is a spectrophotometer that measures nucleic acid and protein concentration in a sample volume of 1-2 µl. The RNA extract (2 µl) was applied to the Nanodrop™ platform and optical density was measured at an absorbance wavelength of 260 nm (A260) to determine concentration of RNA in ng/µl whereby 1 optical density (OD) unit is equivalent to 40 µg RNA. The readings were normalised against the same nuclease-free dH_2O used in the RNA elution step, known as a blank. The quality of the extract was determined by the ratio of OD at A260 to that of 230 nm (A230) and 280 nm (A280). The A260/230 ratio determines contamination with chaotropic salts and ethanol which may have inhibitory effects in downstream enzymatic reactions, while the A260/A280 ratio

indicates the presence of contaminating proteins. The optimal ranges for the A260/A230 and A260/280 are 1.7-2.0 and 1.8-2.0 respectively.

2.1.2 RT-LAMP primer design

This was the first step in the development of the assay and consisted of *in silico* primer sequence selection targeting relevant gene regions and *in vitro* testing of the primers for amplification efficiency and specificity.

A) *In silico* sequence selection

The RT-LAMP amplification is brought about by the action of loop-forming primers, named forward inner (FIP) and back inner (BIP), producing single-stranded regions within the DNA amplicons that can be easily displaced and separated without the need for heat-denaturation and therefore multiple temperatures within a single amplification cycle. This helps to make this technique take-place isothermally. These loop-forming primers are around 40 nucleotides long and designed from 2 neighbouring, but complimentary regions within the desired target gene sequence. Oligomers of this size are prone to contamination as well as self-complementarity, also known as primer self-annealing. When self-annealing occurs, off-target, false positive amplification takes place and the efficiency of amplification of the target is greatly reduced. Further, this technique requires an additional 2 primer pairs: outer primers (F3 and B3), similar to PCR primer pairs which initiate the very first amplification cycle of the LAMP reaction, and loop primers (loopF and loopB). Loop primers hybridise to the loop-region of the amplicons to accelerate exponential amplification of the products. As these primers can only bind to products formed from the first 2 primer pairs, they also aid the specificity of the entire reaction. However, the need for a total of 6 oligomers targeting 1 target gene increases the chances of primer-self-annealing, and *in silico* prediction tools are required to predict the sequence-dependent likelihood of off-target amplification.

Oligonucleotide primers, including published primers and self-designed primers were designed for LAMP according to the recommendations of Notomi et al. (2000) for the outer and inner primer sets and Nagamine et al. (2002) for the Loop primer pair. Four primer sets were assessed including, integrase-1, -2, and integrase-DU179 and published primers targeting p24 (Curtis et al., 2009) (Appendix C). Once primer sequences were obtained and synthesised, each set was applied to the RT-LAMP reaction and tested for

specificity to HIV-1 using restriction digestion analysis. Each of the primer sets are discussed below.

Integrase-1: The integrase-1 primer set was designed against the integrase gene region of the Pol gene of South African HIV-1 isolate 05ZAJF04 (GenBank accession no. GQ872538.1). Primers iF3 and iB3 were obtained using NCBI primer blast (Ye et al., 2012), while the inner (iFIP and iBIP) and loop primers (iLoopF and iLoopB) were self-selected according to the guidelines of Notomi et al. (2000) and Nagamine et al. (2002).

Integrase-2: The second set of primers targeting the integrase region was designed against a sub-type C reference sequence (GenBank accession no. AF286227) listed in the Los Alamos reference sequence database. The total of 6 oligomer sequences, including i2F3, i2B3, i2FIP, i2BIP, i2Loop F and i2Loop B, were selected by assessing for self-hybridisation, using IDT Oligo Analyser (Integrated DNA Technologies, Inc., 2013) and North Western Edu Oligo Calc web-based prediction tools (Kibbe, 2007).

Integrase-DU179: The fourth set of primers was designed against the sequence of isolate DU179 (GenBank accession no. AY043174.1.), for specific RT-LAMP testing on the DU179 culture supernatant sample. For this, LAMP primer design software, Primer Explorer 3 (Fujitsu Ltd.) was used. Primer Explorer 3 generated a list of possible primer sets for the outer (DF3 and DB3) and inner primer pairs (DFIP and DBIP). A single set of primers was selected by a process of elimination by assessing each set for self- and cross-hybridisation using the jPCR software (Kalender, Lee and Schulman, 2011). Loop primers, DloopF and DloopB, were self-selected for the chosen set of primers according to the guidelines of Nagamine et al. (2002) and the final set of 6 primers was assessed using jPCR (Kalender et al., 2011) and for specificity to HIV-1 across the nucleotide database of the nucleotide BLAST alignment tool (Altschul et al., 1990).

P24 by Curtis et al. (2008): The exact pF3, pB3, pFIP, pBIP, pLoopF and pLoopB primer sequences published by Curtis et al. (2008), targeting the p24 gene region of the Gag gene of a sub-type B sequence, for LAMP detection of HIV-1, were tested for reproducibility in the RT-LAMP viral load assay.

B) LAMP and RT-LAMP reaction parameters

Reaction conditions for successful RT-LAMP amplification are well described in the literature (Notomi et al., 2000; Curtis et al., 2008; Goto et al., 2009). Generally, the LAMP reaction mixture was made to contain final concentrations of hydroxy-naphthol blue (HNB) (0.12 mM) (SAS Chemical, Mumbai, India), *BSt* DNA polymerase (8 U) (Lucigen, Middleton, USA), $MgSO_4$ (6 - 10mM) (Sigma-Aldrich, St Louis, USA), deoxyribo-nucleotide triphosphates (dNTP) (1 mM) (Thermo Scientific, Massachusetts, USA), Thermopol reaction buffer (1X) (Lucigen, Middleton, USA) (Appendix B), betaine (0.4 – 1M) (Sigma-Aldrich, St Louis, USA), primer pairs F3 and B3 (0.2 µM); FIP and BIP (1.6 µM); Loop F and Loop B (0.8 µM) and made up to a final volume of 25 µl with sterile, nuclease-free distilled water (Thermo Scientific, Massachusetts, USA). Reaction conditions for RT-LAMP are also well characterised in the literature (Notomi et al., 2000; Curtis et al., 2008, 2009; Buates et al., 2010), and are the same as the above with inclusion of 2.5U - 10U Thermoscript reverse transcriptase (ThermoscriptRT) (Invitrogen, Life Technologies, Carlsbad, USA) or AMV reverse transcriptase (AMV RT) (Promega, Madison, USA) and RNA template as opposed to DNA template is added. Acquisition of RNA template is described above (section 2.1.1). In some cases DNA template was added in the form of integrase clone sequences FV20 and FV23 donated by the HIV Pathogenesis Lab (Department of Molecular Medicine, Wits Med School). The reaction mixture was incubated in a BioRad MJ Mini Thermocycler (BioRad, Hercules, USA) following the parameters outlined in Table 2.1. LAMP and RT-LAMP does not require the use of a thermocycler. Here, the use of a thermocycler instead of heating block was for the convenience of automation of a single temperature change from incubation temperature to inactivation temperature, as well as for the use of a temperature gradient, which aided in reaction temperature optimisation.

Table 2.1 Programme for performing LAMP and RT-LAMP on a thermocycler. The BioRad MJ Mini Thermocycler was used to perform the reactions.

LAMP and RT-LAMP programme	**Temperature (°C)**	**Time**
Incubation	58-65 depending on primer set	1 or 2 hours
Inactivation	85	5 minutes
Hold	4	indefinitely

C) Post RT-LAMP analysis

RT-LAMP amplification detection is simplified by the presence of the reaction by-product, magnesium pyrophosphate ($Mg_2P_2O_7$), which presents as turbidity, a white precipitate or reactant with metal-chelating dyes. Here, precipitation of $Mg_2P_2O_7$ and reaction with HNB dye were used to observe reactions for amplification confirmed by the presence of DNA stem-loop products forming a ladder-like agarose gel electrophoresis pattern. The factors relating to post RT-LAMP analysis are considered below.

i) Magnesium pyrophosphate precipitation

As dNTPs are incorporated into the growing chains of LAMP amplicons, phosphate ions are released which react with $MgSO_4$ to form $Mg_2P_2O_7$. Reactions positive for nucleic acid amplification become turbid due to the formation of $Mg_2P_2O_7$ (Mori et al., 2001). To precipitate the $Mg_2P_2O_7$, as an indicator of a positive reaction, following inactivation of the RT-LAMP reaction at 85°C, reaction tubes were centrifuged at 20000xg (7000-8000 rpm) for approximately 5 seconds, 3 times. The precipitate was observed as a small, white sphere at the bottom of the tube.

ii) Hydroxy-naphthol blue colour change

Hydroxy-naphthol blue dye, violet in colour, is conventionally used as a metal titration indicator, due to the change from violet to blue with increasing presence of metal ions. It is used in the LAMP reaction to detect the change in Mg^{2+} ions that takes place with the accumulation of $Mg_2P_2O_7$ as nucleic acid is amplified. The colour was recorded photographically using the Samsung GT-S5233A camera phone with 2048 x 1536 pixels resolution at time 0 of incubation and following inactivation of the reaction.

iii) Agarose gel electrophoresis

RT-LAMP produces amplicons containing various multiples of stem and loop DNA structures and therefore occurs as a ladder-like banding pattern when analysed by agarose gel electrophoresis. RT-LAMP reaction mixture (5 µl) combined with 6X agarose gel loading buffer (1 µl) (Thermo Scientific, Massachusetts, USA) was loaded onto a 2% TAE agarose gel (Appendix B). DNA was visualised with the aid of GR Green DNA dye (Clare Chemical, Colorado, USA) either added to the liquid gel or to the loading buffer. Electrophoresis was performed at 60-110V at a constant current for 90 – 120 minutes, in 1X TAE buffer (Appendix B). The subsequent gel was viewed and photographed using the

ChemiDoc XRS UV transilluminator (BioRad, Hercules, USA) and QuantityOne gel imaging software supplied with the equipment.

iv) Restriction digestion

The specificity of the reactions was determined by restriction digestion analysis of the RT-LAMP products. Restriction endonucleases cleave DNA at a recognition sequence specific to each endonuclease. By selecting an endonuclease with a restriction recognition site occurring in the expected sequence of DNA, one can assess if that DNA is present in a sample following cleavage to sizes pertaining to the correct recognition sequence, and which can be analysed by agarose gel electrophoresis.

The target sequence, spanning the region between F2 and B2, was entered into NEB Cutter v.2.0 Restriction mapping tool (NEB), which identified a *BSe*XI restriction recognition site. Prior to digestion with *BSe*XI, the LAMP product was purified from the reaction mixture using the GeneJet Purification kit (Thermo Scientific, Massachusetts, USA), according to the manufacturer's protocol (Appendix E). The GeneJet purification procedure is a silica-affinity column procedure similar to that of the viral RNA extraction. Initially, the LAMP reaction mixture was added to a binding buffer which contains GuTc and potassium acetate. The GuTc causes chaotropic disruption of the enzymes and reagents (Blackburn and Gait, 1996) in the LAMP reaction, while the potassium acetate provides a high-salt environment that favours binding of the LAMP-amplified DNA to the silica membrane of the silica affinity column. Centrifugal forces cause the disrupted LAMP reagents to be drawn through the column in solution, collected in a collection tube which was discarded, while the DNA remained bound. This was followed by washing with a wash buffer, also containing GuTc, ensuring any remaining LAMP reagents are disrupted and discarded, along with ethanol which maintains a favourable pH for DNA binding to the column. The column was subsequently dried by centrifugal forces, to remove residual wash buffer. The column was then incubated with Tris-HCl, a low salt buffer, which favours release of the DNA from the silica membrane and promotes DNA longevity during storage. The DNA was then drawn through the column in solution by centrifugal force into a collection tube for storage.

Purified DNA was eluted into Tris-HCl (10 mM) (Thermo Scientific, Massachusetts, USA) at a volume equivalent to the starting volume. The DNA (0.5 µg) was then added to a

digestion reaction mixture containing 3U *BSe*XI (Thermo Scientific, Massachusetts, USA), 1X buffer *BSe*XI (Appendix B) and made up to 20 µl with nuclease-free water (Thermo Scientific, Massachusetts, USA). The mixture was incubated at 65°C for approximately 4 hours and inactivated at 80°C for 20 minutes. The digestion products were analysed by agarose gel electrophoresis as described above, using TBE buffer instead of TAE (Appendix B). Generally, TAE is better for resolution of DNA larger than 1kbp while TBE is preferred for DNA smaller than 1 Kbp. Prior to addition of gel loading dye, digestion products were heated at 65°C in the presence of 1% SDS for 10 minutes as recommended by Thermo Scientific to dissociate any residual enzyme-DNA complexes which will affect gel migration of the digestion product.

2.1.3 RT-LAMP HIV-1 viral load assay optimisation

The optimisation of the assay requires determining the optimal reaction conditions for consistent nucleic acid amplification efficiency, the optimal temperature of incubation, primer design and determining the time-to-positive (TTP). This is done so that the proposed viral load assay performs consistently with respect to external variables for example template RNA quality, patient sample quality and operator-induced variability. The key reagents optimized here were $MgSO_4$ and AMV RT.

A) $MgSO_4$ concentration

Mg^{2+} ions are required for stabilisation of enzymatic reactions. In a PCR reaction, the Mg^{2+} concentration impacts the specificity of primer binding as well as amplification efficiency. Similarly, in a LAMP reaction, the amount of $MgSO_4$ affects the efficiency of *BSt* DNA polymerase activity, interaction with the dNTPs and primers, and also the formation of the magnesium pyrophosphate by-product, the HNB-induced colour change and reverse transcription (Goto et al., 2009). The effect of different concentrations of $MgSO_4$ (0 mM, 5 mM, 6 mM, 7mM, 8mM, 8.5 mM, 9 mM, 10 mM and 12 mM respectively) in the RT-LAMP reaction were tested for effect on amplification efficiency as well as hydroxy-naphthol blue dye interaction and reaction colour development.

B) AMV reverse transcriptase: AMV RT activity is stabilised by free Mg^{2+}ions, and therefore affects HNB dye interaction and reaction colour development. Therefore, the minimum amount of AMV RT at which cDNA synthesis occurred without causing premature colour change from violet to sky-blue had to be determined. Upper and lower

limits of AMV RT, at 10 U and 2 U respectively, were compared for effect on the RT-LAMP reaction colour.

C) Gradient LAMP and Gradient RT-LAMP: LAMP and RT LAMP is generally incubated at 60°-64°C for an hour and inactivated at 80°C-85°C for 5 minutes as suggested in the literature. Some suggest an additional primer-template denaturation step at 95°C for 5 minutes before addition to the reaction mixture (Notomi et al., 2000). Optimum reaction temperature however depends on the specific primer design and characteristics of the template RNA or DNA. For this purpose, LAMP was subjected to a number of temperatures in a single run, known as a gradient LAMP. This was accomplished using the BioRad MJ Mini Thermocycler whereby each row of the thermocycler is heated to a different temperature within a specified range. For this purpose, template positive and template negative LAMP reaction mixtures were incubated at 55°C - 65°C separately, followed by inactivation at 85°C using the same thermocycler. The purpose of assessing each temperature simultaneously was to keep reaction setup and performance of equipment uniform to ensure that any differences observed in reaction efficiency could be attributed to a temperature change alone.

2.1.4 Testing the semi-quantitative capacity of hydroxy-naphthol blue dye detection

Quantification of the amount of template occurring at the start of a nucleic acid amplification reaction can be performed by determining how early the exponential phase of amplification is reached. This is the principal behind quantitative real-time PCR, whereby a higher amount of template or higher viral copy number will amplify faster than a lower amount of template or lower viral copy number. By measuring fluorescent beacons released during amplification, the reaction is monitored in real time to determine the exact point at which an amplification threshold is reached; and then compared to a standard curve to determine an absolute template copy number. Similarly, the turbidity in a LAMP reaction, produced by $Mg_2P_2O_7$, can be measured in real time and compared to reference values as the production of $Mg_2P_2O_7$ is proportional to the accumulation of LAMP product during the LAMP reaction (Mori et al., 2004). It was therefore assessed whether the HNB induced colour-change was sensitive enough to detect the rate of accumulation of $Mg_2P_2O_7$ relevant to different template concentrations or copy numbers. This was performed by the use of a single-self-priming LAMP primer at increasing concentrations in a LAMP reaction. This was based on the rationale that the self-priming primer, identified while

screening the above-mentioned primer sets (section 2.1.2.A), amplifies itself and therefore can be used as template DNA with the added benefit that the primer solution is homogenous and therefore the amount of primer solution added is proportional to the number of primer copies. The LAMP reaction consisted of *BSt Polymerase* (8 U) (Lucigen, Middleton, USA), $MgSO_4$ (6-7 mM)(Sigma-Aldrich, St Louis, USA), Betaine (0.4 M) (Sigma-Aldrich, St Louis, USA), HNB (0.12 mM)(SAS Chemical, Mumbai, India), Thermopol Reaction buffer (1X)(Lucigen, Middleton, USA), dNTPs (1 mM) (Thermo Scientific, Massachusetts, USA) and modified by the inclusion of a self-annealing lamp inner primer, i2FIP at concentrations of 0 µM, 1.6 µM, 3.6 µM and 16 µM, made up to 25 µl in nuclease-free dH_2O (Thermo Scientific, Massachusetts, USA). The remaining five primers required in a regular LAMP reaction as well as template HIV DNA were excluded for control purposes. The reaction was performed in duplicate, incubated at 60°C for 5 minutes and 60 minutes respectively, and inactivated at 85°C for 5 minutes. The reaction colour as well as presence or absence of a $Mg_2P_2O_7$ precipitate was recorded at the start of the reaction, after 5 minutes to record if heating the solution produces a colour change and again after 60 minutes of incubation to observe the end point reaction colour, following nucleic acid amplification. The amount of starting concentration of primer was compared to the amount of accumulated product analysed by agarose gel electrophoresis to confirm the effect of template concentration on the rate of product accumulation after 60 minutes. This was done using a loading dye sensitive to the amount of DNA present, compared to a set of standards. For this, 1 µl of Massruler™ loading dye (Thermo Scientific, Massachusetts, USA) was combined with 5 µl LAMP reaction product or 5 µl Massruler™ ladder (Thermo Scientific, Massachusetts, USA), and loaded onto a 1% TAE gel stained with GR Green gel stain. The gel was imaged as described previously.

Chapter 3

Results

The experimental results of the development of the HIV-1 viral load RT-LAMP assay are presented here. This work includes optimisation of RNA extractions, the design and screening of four RT-LAMP primer sets, the semi-quantitative performance of the hydroxy-naphthol blue dye, followed by performance of the assay on the viral isolate DU179.

3.1 Acquisition of nucleic acid template for development of the RT-LAMP viral load assay

3.1.1 Sample collection

Nucleic acid-based viral load testing conventionally measures copies of viral RNA per ml of plasma as it is an indication of the number of virus particles circulating in the blood of an infected individual. RT-LAMP is also a nucleic-acid based technique. Therefore, HIV-1 RNA was required as the nucleic acid template for the accurate development of the RT-LAMP based viral load assay. In keeping with conventional viral load test procedures, the RNA needed to be extracted from plasma isolated from the whole blood of HIV infected individuals. For this study, 27 HIV infected and 5 HIV negative whole blood samples were collected. The plasma was isolated upon collection and stored for subsequent RNA extraction. WHO 2nd international HIV-1 Standard plasma and HIV-1 subtype C culture supernatant DU179 were obtained as positive controls.

3.1.2 Sample preparation for RNA extraction

Purity of the isolated plasma affects the downstream extraction and subsequent applications of the extracted RNA. The basic procedure of isolation of plasma from whole blood by centrifugation was modified in a number of ways to identify the procedure which gave the best quality RNA. Modifications were compared for the same sample where sufficient sample volume was available. Where this was not possible, modifications were tested across multiple samples. Following extraction of the RNA using the Qiagen viral RNA kit (Qiagen, Hamburg, Germany), the quantity and quality of the RNA was determined by Nanodrop™ spectrophotometry (Thermo Scientific, Massachusetts, USA). Conventionally, RNA and DNA are measured spectrophotometrically by absorbance at 260 nm (A260), protein is measured at 280 nm (A280) and contaminants such as chaotropic salts and ethanol are measured at 230 nm (A230). Therefore, pure quality RNA

displays A260/280 and A260/230 ratios of above 1.8, ideally around 2.0. Plasma samples stored at -20°C, 8 in total, were not suitable for successful RNA extraction as indicated by the low yield and poor quality of the RNA extracted (Table 3.1). Though the 20 000 copy dilution of HIV-1 standard was of a good quality, the yield appeared unexpectedly low (Table 3.1). Similarly, no correlation was observed between yield and viral copy number across the range of dilutions of the HIV-1 standard (Table 3.1). Changing storage of 2 samples from -20°C to -70°C did not improve the quality of the extracts (Table 3.1). However, subsequently collected samples stored at -70°C at first point of use provided RNA of an improved yield and quality than those stored at -20°C (Table 3.1).

Table 3.1 Concentration of RNA extracted from plasma showing the effect of plasma storage temperature on quantity and quality of RNA extracted using the Qiagen Viral RNA mini kit.

	C (ng/µl)		A260/A230		A260/A280	
Sample I.D.	-20°C	-70°C	-20°C	-70°C	-20°C	-70°C
WHO STD	30.7	113.6	1.83	1.6	3.2	2.9
H1	3.9	**82.03**	0.17	0.36	8.68	1.34
N1	3.1	-	0.04	-	-7.1	-
WHO STD 40	29.7	-	0.12	-	3.2	-
WHO STD 400	39.9	-	1.02	-	3.3	-
WHO STD 1000	41.0	-	0.51	-	3.1	-
WHO STD 5000	35.0	-	0.68	-	3.4	-
WHO STD10000	37.9	-	0.90	-	3.0	-
H4	**-**	**158.7**	**-**	**2.27**	**-**	**3.4**
H10	**-**	**155.2**	**-**	**2.40**	**-**	**3.4**

H: HIV-infected plasma; N: HIV Negative plasma; WHO STD: dilution of WHO HIV-1 2nd standard plasma; C: concentration of RNA; A: absorbance in nm. Favourable and unfavourable results indicated in bold and grey respectively. A favourable extract had yield > 80 ng and A260/230 of 1.7-3.0.

The process of viral particle concentration was adopted as a standard procedure based on the rationale that retaining as many of the viral particles from 1 ml of plasma, as opposed to 140 µl of plasma, would be a more accurate representation of the number of RNA copies per ml. Centrifugal concentration was a means of adapting the ideal 1ml sample volume to the Qiagen procedure requiring ideally 140 µl.

The manufacturers of the Qiagen viral RNA extraction kit recommend an optional step of drying the silica column prior to the elution procedure. It was assessed here whether the inclusion or exclusion of this step contributed to the quality and quantity of the extract. No difference was found between inclusion and exclusion of this step (Table 3.2).

Table 3.2 Concentration of RNA extracted from plasma and culture supernatant showing the effect of inclusion (INCL) and exclusion (EXCL) of the dry-column step recommended in the Qiagen viral RNA extraction protocol on purity and quantity of RNA subsequently extracted using the Qiagen Viral RNA mini kit.

	C (ng/µl)		A260/A230		A260/A280	
Sample I.D.	INCL	EXCL	INCL	EXCL	INCL	EXCL
H18	**113.6**	**115.3**	**2.86**	**1.80**	**3.3**	**3.5**
H15	**133.9**	115.6	**2.23**	0.83	**3.29**	3.53
H6	**50.75**	-	**2.59**	-	**4.0**	-
H2	**144.1**	-	**2.89**	-	**3.5**	-
H3	**97**	-	**2.98**	-	**3.4**	-
H5	70.01	-	1.43	-	3.3	-
H10	37.03	-	1.34	-	3.6	-
H7	35.5	-	1.55	-	3.3	-
H19	-	55.7	-	0.53	-	3.81
H20	-	147.44	-	0.58	-	3.27
DU179	-	**124.1**	-	**2.64**	-	**3.28**

Plasma stored at -70°C prior to centrifugal concentration. H: HIV-infected plasma; N: HIV negative plasma; C: concentration of RNA; A: absorbance in nm; DU179: RNA extracted from culture supernatant instead of patient plasma. Favourable and unfavourable results indicated in bold and grey respectively. A favourable extract had yield > 80 ng and A260/230 of 1.7-3.0.

Plasma, though free of cells, contains various enzymes and other proteins that may affect the extraction process. The use of serum separator tubes (SST) was tested as a means of pre-cleaning plasma prior to viral particle concentration. In almost all cases, the use of SST improved the purity of the resulting RNA compared to the same samples processed without these tubes (Table 3.3).

Through the above performed tests and modifications it was established that the optimal procedure for extracting RNA from plasma necessitated SST separation of whole blood, followed by high speed centrifugation of 1 ml of isolated plasma. The viral pellet should then be resuspended in 200 µl PBS as starting material for the Qiagen Viral RNA mini procedure and then processed according to the manufacturer's protocol, but with exclusion of the optional step of drying out the column prior to elution. All plasma and RNA should be stored at -70°C.

Table 3.3 Concentration of RNA extracted from plasma showing the effect of passing whole blood or plasma through serum separator tubes (SST) prior to performing centrifugal concentration on purity and quantity of RNA subsequently extracted using the Qiagen Viral RNA mini kit excluding the dry-column step.

	C (ng/µl)		A260/A230		A260/A280	
Sample I.D.	SST	no SST	SST	No SST	SST	No SST
H12	25.7	120.07	0.12	0.6	3.45	3.13
H16	**83.5**	74.4	**2.17**	0.16	**3.2**	3.6
H17	**113.7**	77.0	**2.59**	0.17	**3.2**	3.8
H18	**89.46**	**115.3**	**1.79**	**1.80**	**3.3**	**3.5**
N4	**92.5**	47.0	**1.71**	0.60	**3.4**	**3.5**
H13	**150.5**	**-**	**1.97**	**-**	**3.3**	**-**
H14	135.1	**-**	0.57	**-**	3.45	**-**

H: HIV-infected sample; N: HIV negative sample; C: concentration of RNA; A: absorbance in nm. Favourable and unfavourable results indicated in bold and grey respectively. A favourable extract had yield > 80 ng and A260/230 of 1.7-3.0.

3.2. RT-LAMP HIV-1 viral load assay primer design

The unique primer design of RT-LAMP allows the technique to amplify nucleic acid isothermally. Six primers are required across 4 regions of the gene targeted for amplification. Four of the 6, being the 2 outer primers (F3 and B3) and 2 loop primers (Loop F and Loop B) are similar in length to PCR primers. The remaining 2 inner primers (FIP and BIP) each span up to 40 nucleotides in length, each corresponding to 2 adjacent regions within the target gene and produce the loops in the repeated stem-loop amplification products. For the viral load assay, RT-LAMP primers were designed against 2 target gene regions of HIV-1, p24 and integrase, respectively. These gene regions are targeted by commercial real-time RT-PCR viral load assay kits (Abravaya et al., 2003; Schumacher et al., 2007). A total of 4 sets of the 6 RT-LAMP primers were designed and assessed for suitability for the RT-LAMP HIV-1 assay, 1 targeting p24 and the other 3 targeting integrase. Primer screening involved performing RT-LAMP with each primer set in the presence of patient-obtained HIV-1 template and negative controls (no RT and no-template controls), and where available, positive controls: WHO standard RNA, DU179 culture supernatant RNA and cloned integrase DNA. Amplification products were assessed for specificity to HIV-1 by restriction digestion analysis.

3.2.1 p24 primer set

Primers targeting the p24 gene region of the HIV-1 Gag gene, previously designed for an RT-LAMP diagnostic assay (Curtis, Rudolph and Owen et al., 2009), were tested to see if the published results could be replicated for use in the viral load assay. RT-LAMP was performed across a range of incubation temperatures from 58°C to 64°C (Fig. 3.1). Although Curtis, Rudolph and Owen (2008) established an optimal temperature for these primers, the reaction conditions differed as they did not use the HNB detection method. The temperature range applied was ascertained from LAMP literature and recommended conditions for *BSt* Polymerase activity. RNA extracted from patient plasma was applied as template, so as to simulate conventional viral load testing. A patient initiating treatment was selected as it was expected that such a patient would have a detectable viral load although this was not confirmed as viral load testing was unavailable as viral load is not done at baseline of treatment initiation according to the South African ART treatment guidelines (Department of Health, 2013).

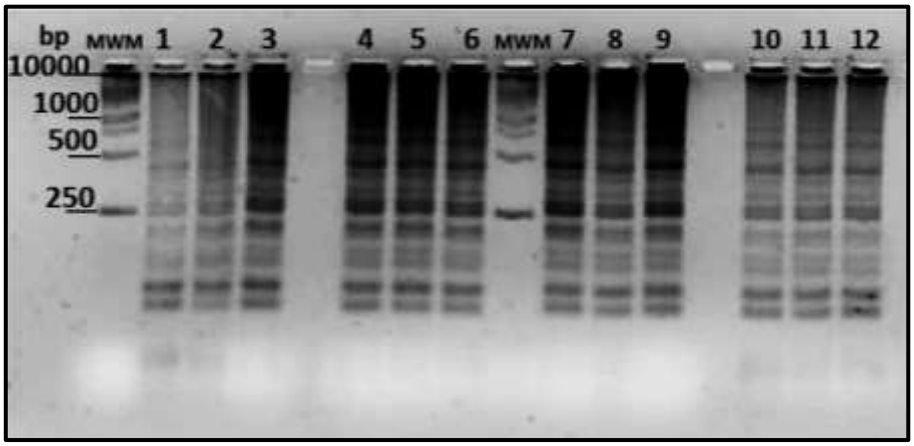


Figure 3.1 Gradient RT-LAMP performed on p24-1 primer set to test primer performance when RT-LAMP is carried out for 60 minutes at 58°C (lanes 1-3); 60.4°C (lanes 4-6); 62.8°C (lanes 7-9); 64°C (lanes 10-12). RT-LAMP was performed on RNA (82 ng) obtained from HIV seropositive patient H1 initiating treatment (lanes 1,2,4,5,7,8,10 and 11); Controls included no RT (lanes 2,5,8 and 11)and no template controls (lanes 3,6,9 and 12). For the negative controls water replaced RT and RNA respectively. MWM: 1Kb GeneRuler™ (bp) (4 µl). 2% Tris-Boric Acid-EDTA agarose gel stained with GelRed nucleic acid gel stain. Electrophoresis performed at 60V for 60 minutes. Lanes contain 5 µl reaction products combined with 1 µl GelRed loading buffer.

With agarose gel electrophoresis, amplification was observed at all temperatures (Fig. 3.1), evident as a ladder-like banding pattern, made up of products as small as 200 bp up to 10 000 bp sized products containing multiple stems and loops. All the bands were observed to be identical including that of the no template controls (Fig. 3.1). This means that the observed amplification on the gel represents that of background, off-target amplification and not amplification of HIV RNA, indicating that further optimisation was required.

To aid in determining the source of off-target amplification in the negative controls, it was tested whether exclusion of certain reagents prevented amplification in no template control experiments (Fig. 3.2A). External sources of contamination were ruled out as basis for the off-target amplification as exclusion of RT, betaine, HNB dye, reaction buffer and nuclease-free water did not prevent off-target amplification (Fig. 3.2A).

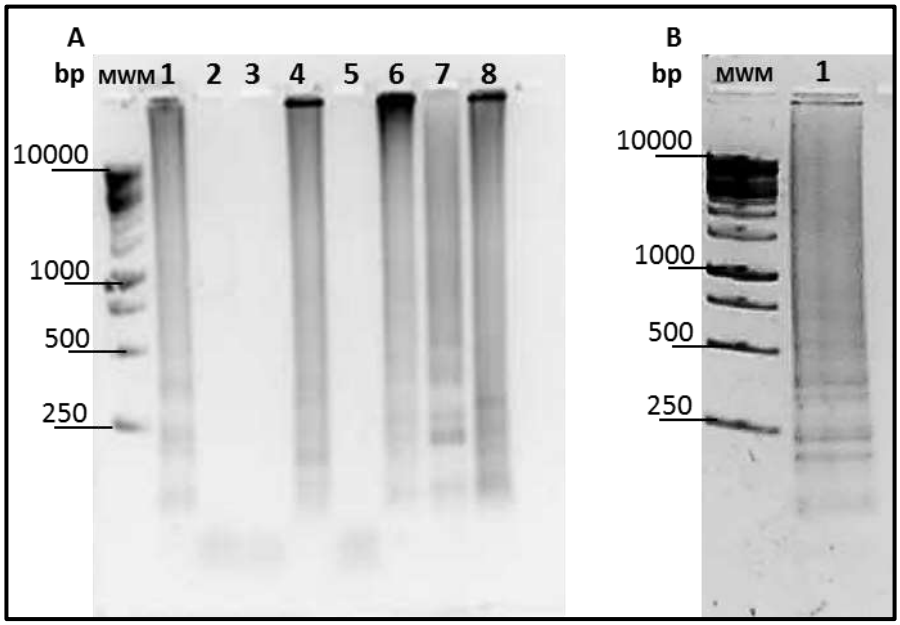


Figure 3.2 RT-LAMP performed with primer set p24-1 on no template controls to determine source of persistent off-target amplification products occurring in reactions primed by the p24-1 primer set. A) The following reagents were excluded from the RT-LAMP reaction: RT (lane 1), *Bst* polymerase (lane 2), dNTPs (lane 3), Betaine (lane 4), $MgSO_4$ (lane 5), HNB (lane 6), reaction buffer (lane 7), water (lane 8). Reaction performed at 63°C for 60 minutes. B) RT-LAMP using p24 primers in a no template control using new stocks of *BSt* Pol, dNTPs and $MgSO_4$, reaction performed 63°C for 40 minutes. A) and B) 1.2 % Tris-Boric Acid-EDTA gel stained with GelRed nucleic acid gel stain. MWM: 1Kb GeneRuler™ (2 µl). Electrophoresis performed at 97 V for 60 minutes (A) and 102 V for 60 minutes (B). Lanes contain 5 µl reaction products combined with 1 µl 6X loading dye.

Here, amplification was only absent when reagents essential to LAMP, such as *BSt* polymerase, deoxy-nucleotide-triphosphates (dNTP) and $MgSO_4$, were excluded (Fig. 3.2A). This was confirmed by the persistence of off-target amplification with the use of new reagent stocks (Fig. 3.2B). Reducing the reaction time from 60 minutes (Fig. 3.2A) to 40 minutes (Fig. 3.2B), did not prevent off-target amplification. Overall, the primers designed by Curtis et al. (2008), were deemed unsuitable for use in the RT-LAMP viral load assay due to the presence of off-target amplification which could later lead to false-positive results in tested samples.

3.2.2 Integrase-1 primer set

The 2 gene regions targeted by conventional real-time PCR viral load assays are p24 and integrase (Abravaya et al., 2003; Schumacher et al., 2007). In keeping with this convention, a second set of primers was designed against the integrase region of the *Pol* gene (Fig. 3.3). RT-LAMP primers were designed according to the recommendations of Notomi et al. (2000) and Nagamine et al. (2002) for LAMP and loop primers respectively. Initially, RNA extracted from WHO 2nd standard for HIV-1 was used at various dilutions

to test the primers and observe if the reaction could distinguish the different viral loads. Integrase-1 primers were designed against sub-type C as this is the predominant sub-type in South Africa (van Harmelen et al., 1999). Considering that the WHO standard is a sub-type B isolate, RNA obtained from an ART-naïve patient was also applied in keeping with the South African viral load context. Similarly to the p24 primers, amplification was observed in the negative controls including no RT, no template and negative template controls (Fig. 3.3).

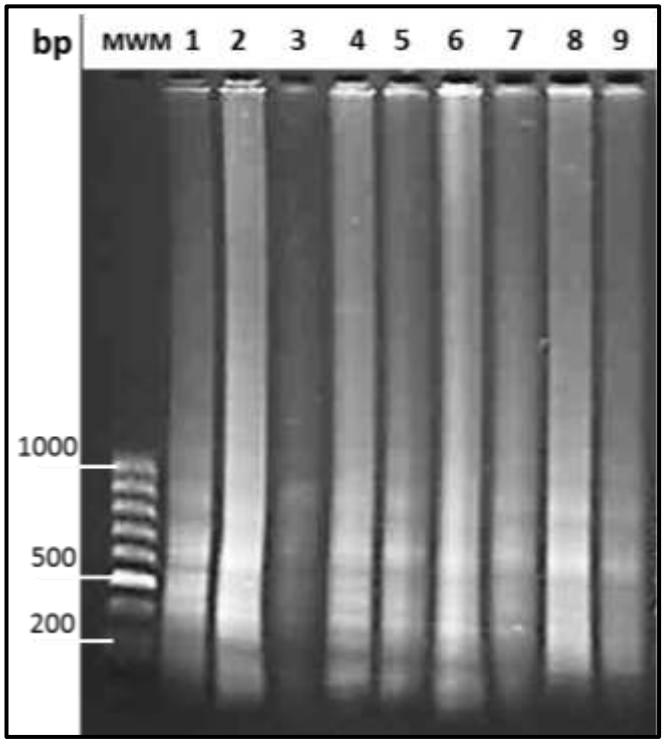


Figure 3.3 RT-LAMP performed using the integrase-1 primer set, carried out at 60°C for 60 minutes. The following sources of template RNA were used: WHO 2nd standard HIV RNA, 363 000 copy dilution (3 ng)(lanes 1-2); WHO 2nd standard HIV RNA 10 000 copy dilution (38 ng) (lanes 3-4); RNA (82 ng) from patient H1 plasma (lanes 5-6); RNA from HIV negative individual N1 (3 ng) (lanes 7-8); no template control (lane 9); no RT control(lanes 2, 4 and 6).1.2% Tris-Boric Acid-EDTA agarose gel stained with GelRed nucleic acid gel stain. MWM: 100 bp GeneRuler™ (3 µl). Electrophoresis performed at 100V for 60 minutes. Lanes contain 5 µl reaction products combined with 1 µl GelRed loading buffer.

As was done in response to off-target amplification observed with the use of the p24 primers, reagents RT, *BSt* Pol, dNTPs, betaine and $MgSO_4$, were checked for contamination by excluding them from the reaction (Fig. 3.4).

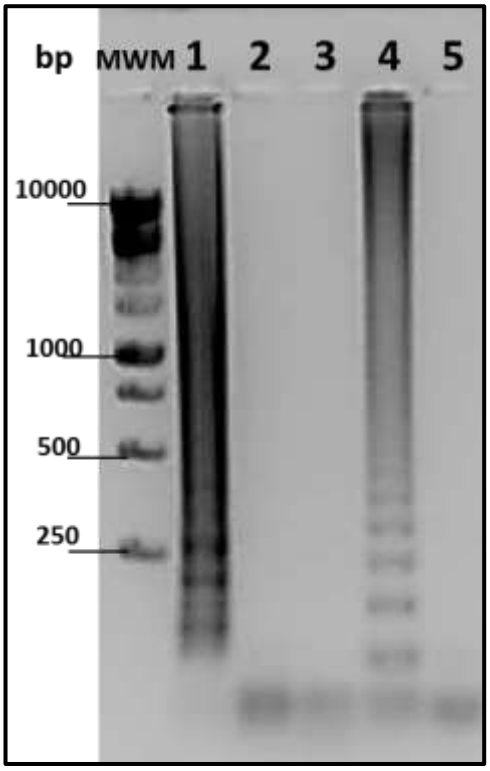


Figure 3.4 RT-LAMP performed with integrase-1 primer set to determine source of persistent off-target amplification in no template controls, carried out at 63°C for 60 minutes. The following reagents were excluded from the RT-LAMP reaction. RT (lane 1), *Bst* polymerase (lane 2), dNTPs (lane 3), betaine(lane 4), $MgSO_4$ (lane 5). 1.2% Tris-Boric Acid-EDTA agarose gel stained with GelRed nucleic acid gel stain. MWM: 1Kb DNA ladder (3µl). Electrophoresis performed at 97V for 60 minutes. Lanes contain 5 µl reaction products combined with 1 µl 6X loading buffer.

Amplification was not hindered by the exclusion of RT or betaine (Fig. 3.4). It was therefore assumed that the source of off-target amplification could be self-annealing primers. This is supported by the observation that the source is DNA, since amplification occurred in the absence of RT (Fig. 3.3 lanes 2, 4, 6; Fig. 3.4 lane 1). Increasing the reaction temperature from 60°C (Fig. 3.3) to 63°C (Fig. 3.4) did not prevent off-target amplification.

3.2.3 Primer set 3, targeting integrase (Integrase II)

A set of primers was designed against the integrase region of the *Pol* gene, against an HIV-1 sub-type C reference sequence (GenBank accession no. AF286227). To screen these primers, RT-LAMP reactions were performed on RNA from ARV naive and treated individuals (Fig. 3.5).

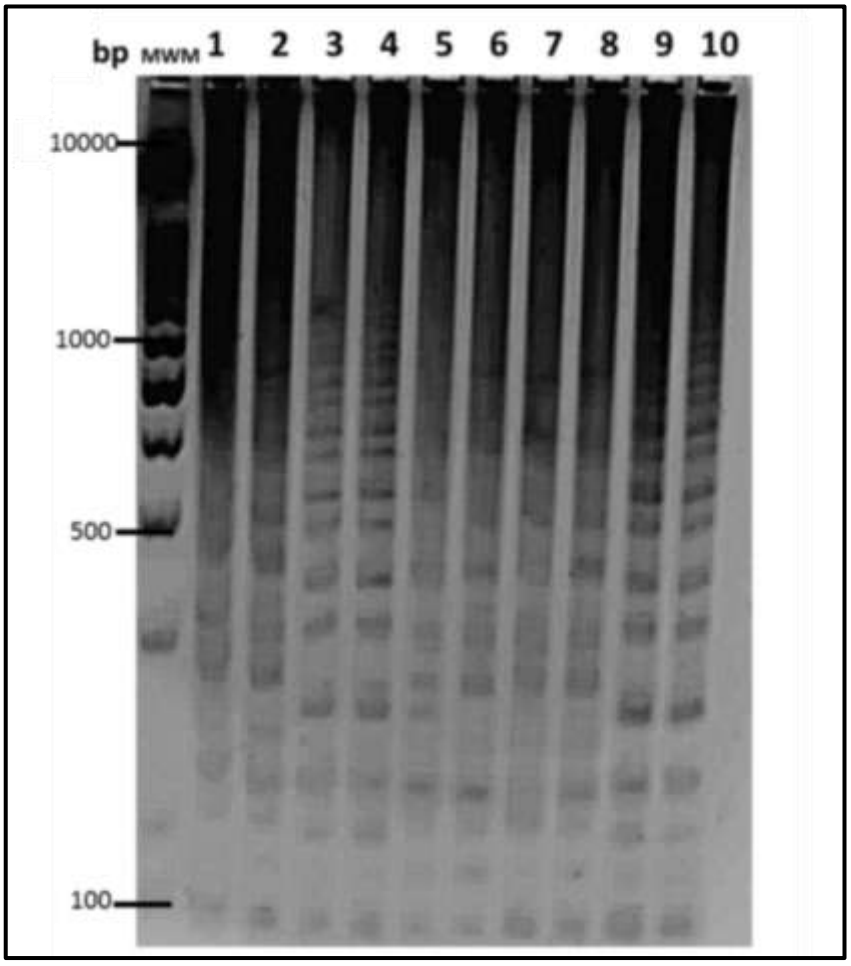


Figure 3.5 RT-LAMP performed with the integrase-2 primer set, carried out at 60°C for 60 minutes. The following sources of RNA template were used: Treated HIV positive patient (144.41 ng) (lane 1 and 2), treated HIV positive patient H3 (97 ng) (lane 3 and 4), HIV patient initiating treatment H5 (70 ng) (lanes 5 and 6), HIV patient initiating treatment H10 (37 ng) (lanes 7 and 8). No RT controls (lanes 2,4,6,8 and 10) and no template controls (lanes 9 and 10) were included. 2% TBE agarose gel stained with GR Green nucleic acid gel stain. MWM: 10 Kb DNA mass ruler (5 µl). Electrophoresis performed at 90V for 60 minutes. Lanes contain 5 µl of reaction product combined with 1 µl 6X loading buffer.

LAMP reactions were performed on cloned integrase DNA (Fig. 3.6). This was done as a control experiment to eliminate any problems that may arise from the use of patient-derived RNA as well as single-step reverse transcription. As observed with the previous 2 primer designs, off-target amplification was evident in the no template control and HIV-negative control reactions (Fig. 3.5 lanes 9-10; Fig. 3.6 lanes 3-4).

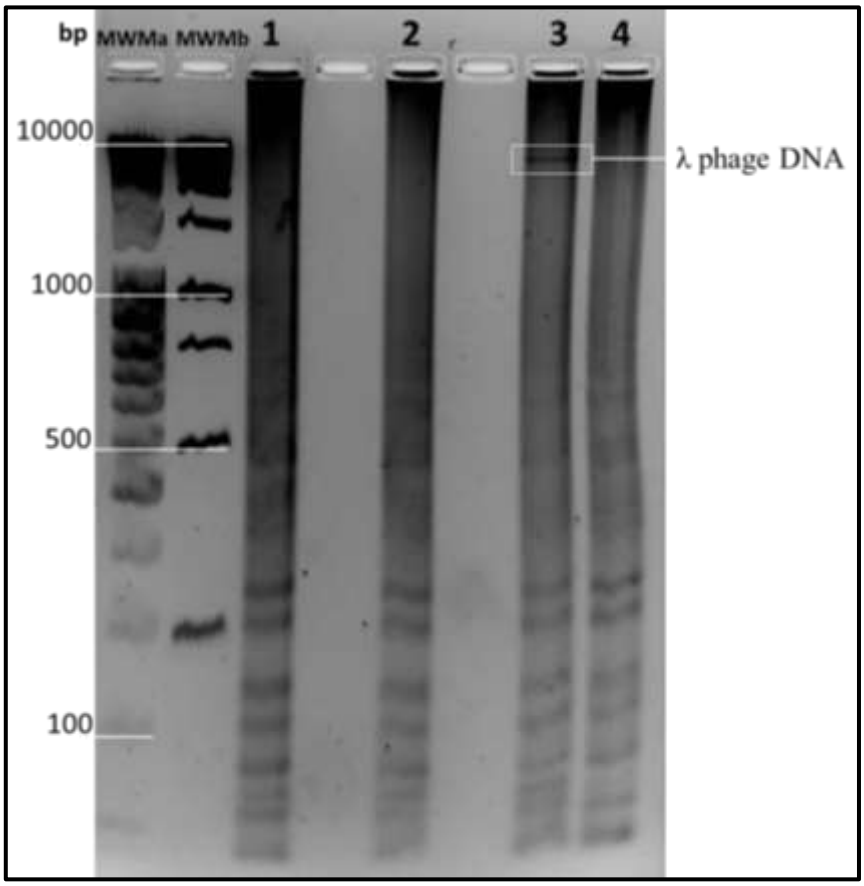


Figure 3.6 LAMP performed using integrase-2 primer set at 60°C, for 60 minutes. Cloned integrase sequences FV20 (lane 1), and FV23 (lane 2) were used as template DNA. Lambda phage DNA (λ) was used as template in a negative control reaction (lane 3) along with a no template control (lane 4). 2% TBE agarose gel stained with GR Green nucleic acid gel stain. MWMa: DNA Mass Ruler (10kb) (3 µl); MWMb: 1Kb GeneRuler™ DNA molecular weight marker (3 µl). Electrophoresis performed at 100V for 60 minutes. Lanes contain 5 µl of reaction product combined with 1 µl 6X loading buffer.

The use of negative control DNA, such as lambda phage DNA, aided in determining the source of off-target amplification. As seen in Figure 3.6 (lane 3), the intact presence of the HIV negative DNA reveals that the off-target amplification occurring in that reaction was not a result of off-target primer-template hybridisation. It was suspected that the off-target amplification was once again caused by primer dimerization. To identify this, LAMP was performed on no template controls with different combinations of the 4 primers, F3, B3, FIP and BIP based on the assumption that no amplification would be observed in the absence of the primer(s) engaging in dimerization (Fig. 3.7).

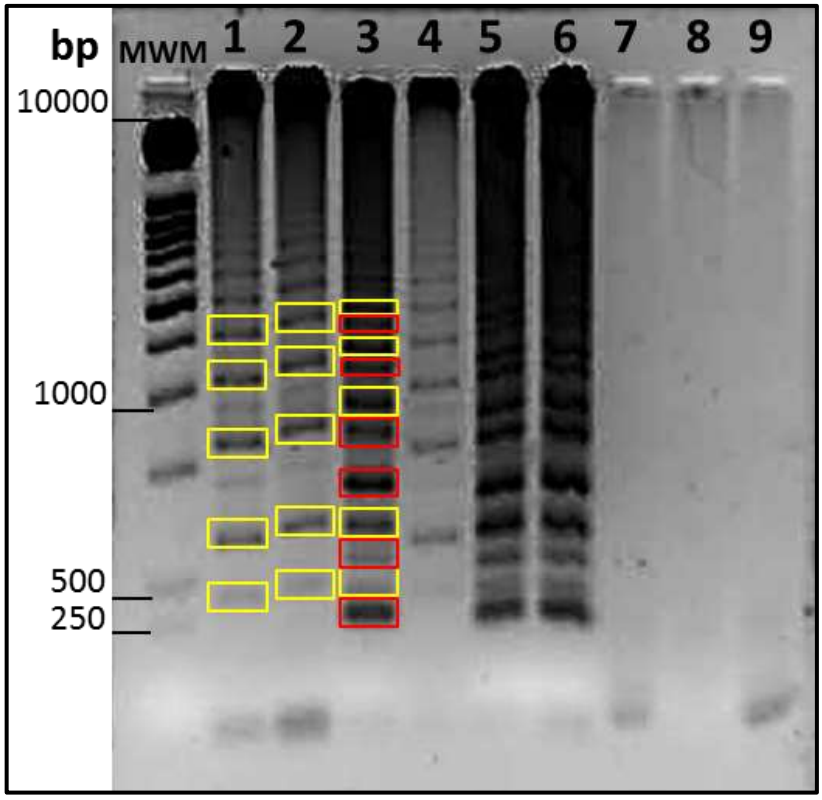


Figure 3.7 LAMP performed on no template controls using various combinations of integrase-2 primers F3, B3, FIP and BIP to determine the source of off-target amplification carried out at 60°C for 60 minutes. Primer combinations: F3 and FIP (lane 1); B3 and FIP (lane 2); FIP and BIP (lane 3); F3, B3 and FIP (lane 4); F3, FIP and BIP (lane 5); B3, FIP and BIP (lane 6); F3 and BIP (lane 7); B3 and BIP (lane 8); F3, B3 and BIP (lane9). 2% TBE agarose gel stained with GR Green nucleic acid gel stain. MWM: 1Kb GeneRuler™ DNA ladder (4 µl). Electrophoresis performed at 100V for 60 minutes. Lanes contain 5 µl of reaction product combined with 1 µl 6X loading buffer. Yellow: products of suspected i2FIP homo-dimerization, Red: products of suspected i2FIP-i2BIP hetero-dimerization.

LAMP amplification products were evident in all reactions containing primer FIP (Fig. 3.7, lanes 1-6) which suggests that integrase-2 primer FIP (i2FIP) was responsible for the presence of off-target amplification using the integrase-2 primer set. Lane 3 (Fig. 3.7) consisting of the 2 40-meric primers, i2FIP and i2BIP, appears to display the same electrophoresis pattern evident in the presence of primers, i2F3 and i2B3 (Fig. 3.7, lanes 1, 2 and 4), superimposed with other bands, even though neither of the smaller primers were included in this reaction. This suggests that the products formed in the presence of i2F3, i2B3 and i2FIP (yellow), are a result of i2FIP homo-dimerization, while the other bands present in lane 3 pertain to i2FIP-i2BIP hetero-dimerization (red) (Fig. 3.7).

The original LAMP reaction included heating primer-template solutions to 95°C before addition to the reaction mixture. This was done to determine if the added heating step

would improve initial primer-target hybridisation and prevent the observed primer homo- and hetero-dimerization (Fig. 3.8).

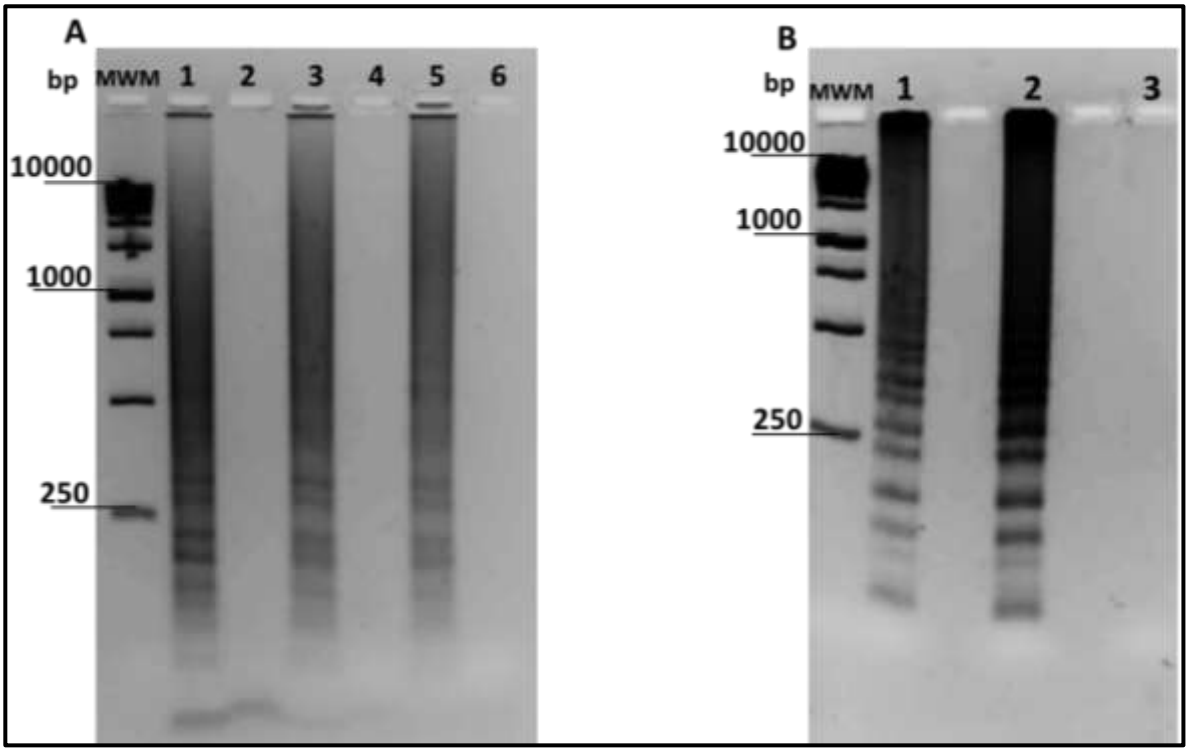


Figure 3.8 Testing effect of denaturing template and primers at 95°C prior to a LAMP reaction. LAMP carried out at a temperature gradient using integrase-2 primers. A) Cloned integrase FV23 template DNA (lanes 1,3 and 5) and no template controls (lanes 2,4 and 6) were combined with the 6 primers and heated at 95°C before addition to the LAMP reaction mixture performed at 55°C (lanes 1 and 2), 58°C (lanes 3 and 4) or 64°C (lanes 5 and 6) respectively for 60 minutes. B)Cloned integrase DNA template (lane 1), a lambda phage DNA negative control (lane 2) and no template control (lane 3)were combined with the 6 primers and heated at 95°C before addition to the LAMP reaction mixture and incubated at 60°C for 60 minutes. MWM: 1Kb GeneRuler™ (4 µl). 2% TBE agarose gel stained with GR Green nucleic acid gel stain. Electrophoresis performed at 100V for 60 minutes. Lanes contain 5 µl of reaction product combined with 1 µl 6X loading buffer.

Initially, amplification was only observed in the reactions containing integrase-DNA template (Fig. 3.8 A, lanes 1, 3 and 5) but not in the water blank (Fig. 3.8 A, lanes 2, 4 and 6). However a repeat of the same reaction revealed amplification in the presence of HIV-negative control template lambda phage DNA as well (Fig.3.8 B, lane 2) with both reactions producing identical electrophoresis bands, indicating that the amplification was off-target, which could not be prevented by an additional heating step or alternative incubation temperature.

3.2.4 Primer set 4 targeting HIV-1 isolate DU179 integrase (Integrase-DU179)

A set of primers was designed using new online prediction tools. To eliminate the problems of screening primers using RNA obtained from patient plasma, a homogenous HIV-1 culture supernatant, isolate DU179, was acquired as a source of viral RNA from the Lynn Morris HIV Virology Lab, NICD, SA. The DU179 isolate was previously sequenced and primers were therefore designed for this specific isolate (GenBank accession no. AY043174.1.). The sequence was entered into the LAMP primer design software, primer explorer version 3. A combination of primer sets was provided from the software. Each set of primer sequences was analysed for primer-dimerisation on jPCR. No primer-dimers could be predicted for the following set and was therefore sent for oligo synthesis.

Gradient RT-LAMP was performed to test the primers at a temperature range of 59°C to 62°C, for 60 minutes. Amplification was observed only in the presence of HIV DU179 template RNA (Fig. 3.9 lanes 7-9), but not in the no RT control (Fig. 3.9, lanes 4-6) and no template controls (Fig. 3.9, lanes 1-3).

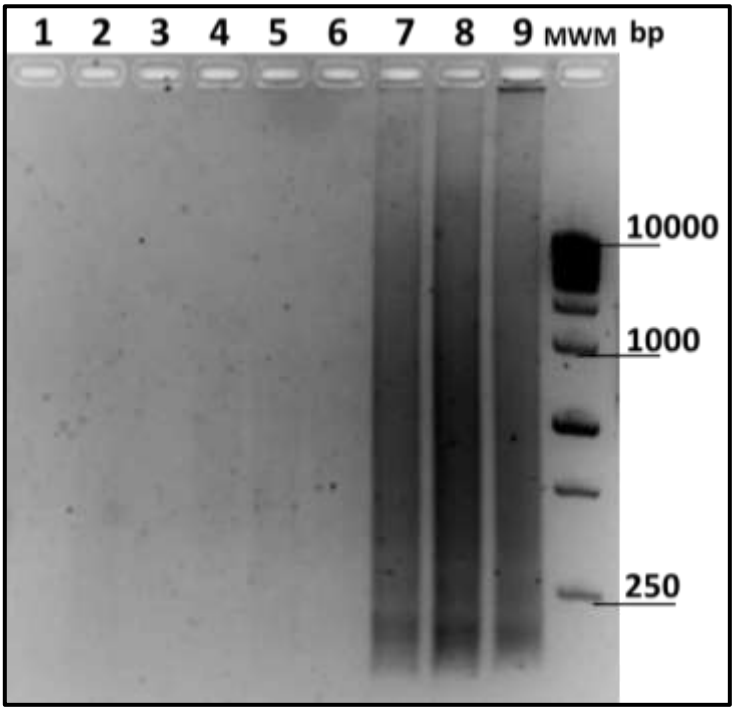


Figure 3.9 Gradient RT-LAMP to test integrase-3/DU179 primers using HIV-1 isolate DU179 culture supernatant RNA (8708 nt) (lanes 4-9). RT-LAMP was performed for 60 minutes at: 59°C (lanes 1,4 and 7); 60°C (lanes 2,5 and 8); 62°C (lanes 3,6 and 9)and include no RT (lanes 4-6) and no template controls (lanes 1-3). MWM: 1 kb DNA ladder (4 µl). 2%TAE agarose gel stained with GR Green gel stain. Electrophoresis performed at 110V for 60 minutes. Lanes contain 5 µl of reaction product combined with 1 µl 6X loading buffer.

3.2.5 Restriction digestion analysis to determine specificity of primers to HIV-1

Amplification products produced by RT-LAMP primed with the DU179-integrase primer set were analysed for specificity to HIV-1 by restriction digestion analysis. *BSe*XI was chosen for its recognition site spanning the gap between the F2 and F1 region of the forward inner primer, producing a 49 base pair stem-loop fragment and a 126 bp fragment spanning the F1 stem and downstream target. Agarose gel electrophoresis was performed to observe for these fragments (Fig. 3.10).

Digestion with this enzyme changed the ladder-like electrophoresis pattern (Fig. 3.10, lane 2) to 2 large bands (Fig. 3.10, lane 1), one around the expected size of 126 bp and another slightly larger, possibly as a result of incomplete digestion of large multiple stem-loop structures, suggesting that the amplification products are HIV specific. The integrase-DU179 primer set was therefore selected for use in the viral load assay.

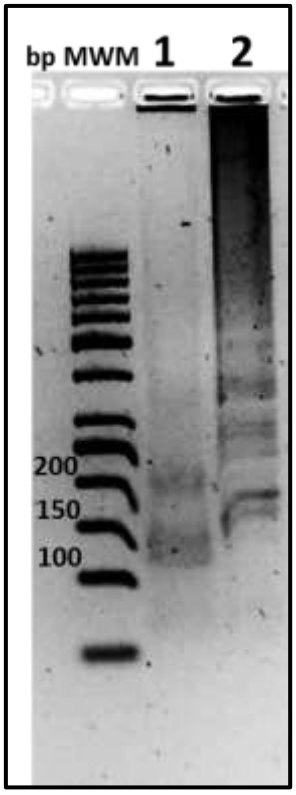


Figure 3.10 *BSe*XI restriction digestion of RT-LAMP products primed by DU179-integrase primers. Digestion products (lane 1) and undigested RT-LAMP reaction.2% TBE agarose gel stained with GR Green nucleic acid gel stain. MWM: 50 bp DNA GeneRuler (4 µl). Electrophoresis performed at 90V for 90 minutes. Lanes contain 5 µl of digestion and RT-LAMP reaction product respectively.

3.3 Optimisation of reagents required for the RT-LAMP HIV-1 viral load assay

3.3.1 Reverse Transcriptase

It was found that in the presence of reverse transcriptase, the reaction changed colour from violet to blue prior to the start of the reaction, producing a false-positive result (Fig. 3.11). 10 mM$MgSO_4$ was used in these reactions as reactions usually remain violet above 9 mM $MgSO_4$ (Goto et al., 2009). Yet this did not prevent the RT-induced colour change (Fig. 3.11 and 3.12).When the amount of RT added to the reaction was reduced from 10 units to 2 units, the reaction colour remained violet (Fig. 3.14).

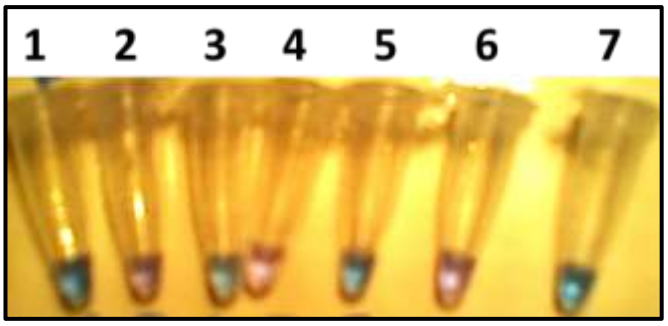


Figure 3.11 The effect of reverse transcriptase concentration on available $Mg^{2+}$and reaction colour in p24-1 primed RT-LAMP reactions containing hydroxy-naphthol blue dye. Tubes1,3,5 and 7: 10U AMV RT; Tubes 2,4, and 6: 0U AMV RT; Tubes 1-7: 10mM $MgSO_4$.

As previously identified during the primer design steps (section 3.2), use of the p24-1 primer set produced off-target amplification (Fig. 3.1-3.3). Although amplification was evident in the no RT controls, these reactions remained violet in colour (Fig. 3.11) indicating that reactions which turned blue, including reactions performed with HIV RNA template and no template controls, did so as a result of the presence of RT. This was supported by the observation of reaction mixtures turning blue at the start of the reaction (Fig. 3.12). Also no difference could be observed in the reaction colours after 60 minutes (Fig. 3.12) which suggests that amplification by primer self-annealing did not produce enough Magnesium pyrophosphate ($Mg_2P_2O_7$) to produce a colour change from violet to blue and therefore, in the context of the viral load assay, off-target amplification will come across as undetectable.

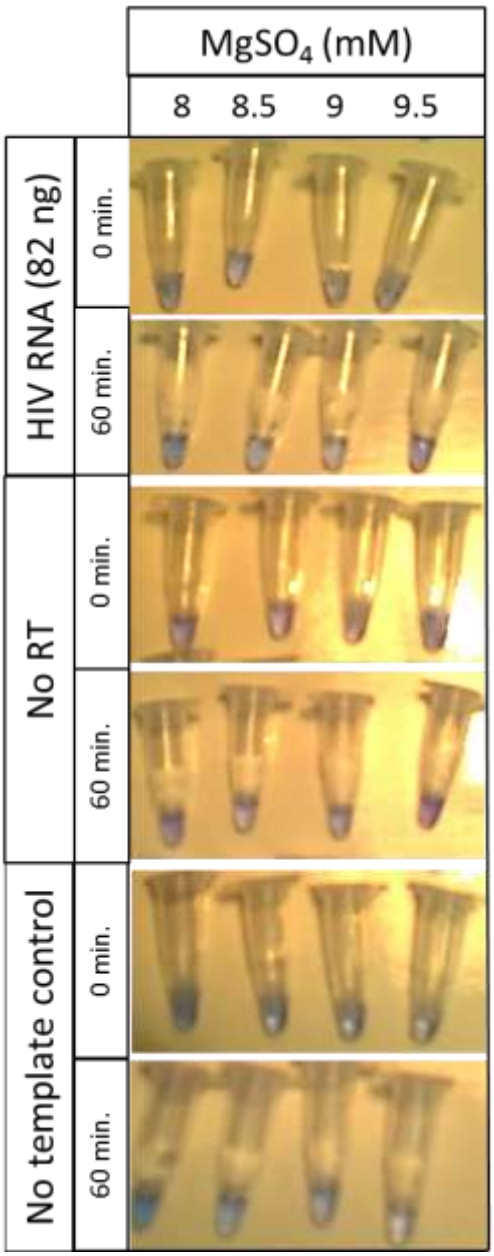


Figure 3.12 The effect of $MgSO_4$ concentration (mM) on HNB-induced reaction colour in the presence (HIV RNA and no template controls) and absence of RT (no RT control). $MgSO_4$ was added to reactions at a final concentration of 8, 8.5, 9 and 9.5 mM respectively. The reaction was performed using p24-1 primer set for 60 minutes at 63°C.

3.3.2 Magnesium sulphate ($MgSO_4$)

The dye hydroxy-naphthol blue chelates with free metal ions of calcium and magnesium in solution, changing from violet to sky blue. In the RT-LAMP reaction, the dye interacts with the free Mg^{2+} ions produced during the reaction between $MgSO_4$ and the phosphate group released during dNTP incorporation into the extending amplification products. It was previously determined by Goto et al. (2009) that the amount of $MgSO_4$ added to the reaction affects the behaviour of HNB, whereby too little $MgSO_4$ causes a premature colour change. It was confirmed earlier that off-target amplification does not produce a

colour change (Fig. 3.12). Therefore, a colour change produced by a no template control indicates an unstable dye interaction. No template control reactions were performed to test the lowest concentration of $MgSO_4$ required for stable dye interactions (Fig. 3.13).

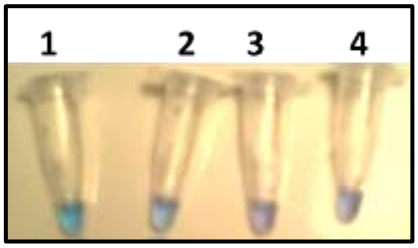


Figure 3.13 The effect of MgSO4 concentration on colour of a LAMP reaction containing hydroxy-naphthol blue dye and no template DNA: 1) 0 mM $MgSO_4$; 2) 5 mM 3) 6 mM 4) 7 mM.

The results of Goto et al. (2009) were confirmed in this study (Fig. 3.13) and it was determined that the HNB reaction remained stable with the use of 6-8mM $MgSO_4$, since at these concentrations the reaction remained violet in colour in the absence of RT (Fig. 3.12 and 3.13 tubes 3-4), the correct colour for a no-template negative control. Goto et al. (2009) determined that $MgSO_4$ above 9 mM prevented a colour change in positive reactions and therefore 8 mM was selected as the highest acceptable concentration. The blue colour of the reaction without $MgSO_4$ is indicative of the colour change that takes place once free Mg^{2+} ions are consumed.

Following determining stable concentrations of $MgSO_4$, RT LAMP was performed using 7mM $MgSO_4$ to determine the effect of reducing RT from 10 Units to 2 Units on the HNB-induced reaction colour (Fig. 3.14). The integrase-2 primer set, which produces off-target amplification, was used and no colour change was observed, which is the expected result for stable dye interactions following off-target amplification (Fig. 3.14). Therefore, the optimal RT amount was accepted as 2 units.

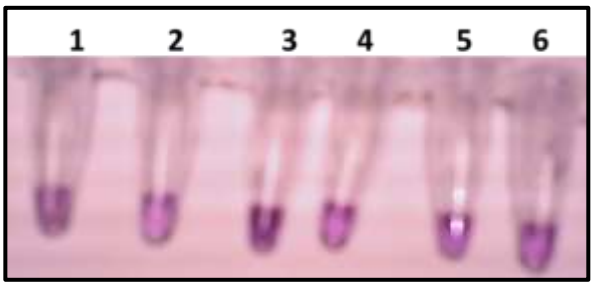


Figure 3.14 The effect of reverse transcriptase (RT) concentration on reaction colour of integrase-2 primed RT-LAMP reaction containing Hydroxy-naphthol blue dye. Reactions contain 7 mM $MgSO_4$ and 2 U AMV RT. RT-LAMP was performed at 60°C. HIV-infected plasma-derived RNA (tubes 1 and 3) and HIV standard RNA (tubes 2 and 4) were added to the RT-LAMP reaction alongside RT negative (tubes 3, 4 and 6) and no template controls (tubes 5 and 6).

3.3.3 Hydroxy-naphthol blue

As seen with the use of the first 3 primer sets, the reaction colour remained violet for all reactions, despite the definite presence of amplification on the agarose gels. Given that self-primed off-target amplification is not as efficient as amplification as a result of hybridisation with the target template, it was hypothesised that the colour change would only take place once a threshold of magnesium pyrophosphate was produced. This was tested by increasing the amount of self-annealing primer FIP from the integrase-2 set (i2FIP) (section 3.2.3), expecting to speed up the accumulation of magnesium pyrophosphate to produce a colour change (Fig. 3.15-3.18).

Reactions containing lower amounts of i2FIP (0-1.6 µM) did not change colour after 60 minutes whereas reactions with higher amounts of i2FIP (3.6-16 µM) did change from violet to blue (Fig. 3.15). The colour change was solely indicative of rate of increase in amplification, and not as a result of a premature colour change or heating the reaction mixture, as all reactions remained violet at the start of the reaction as well as 5 minutes into the reaction respectively (Fig. 3.15).

To confirm that production of the $Mg_2P_2O_7$ by-product only produced a colour-change after reaching a threshold of amplification, LAMP reactions performed with different amounts of the self-annealing i2FIP primer were analysed for by-product (Fig. 3.16).There was no magnesium pyrophosphate precipitate at the start of the reaction but was present in both the violet (1.6 µM i2FIP, Fig. 3.15 and 3.16) and blue tubes (3.6 µM, Fig. 3.15 and 3.16) after 60 minutes of RT-LAMP incubation (Fig. 3.16).

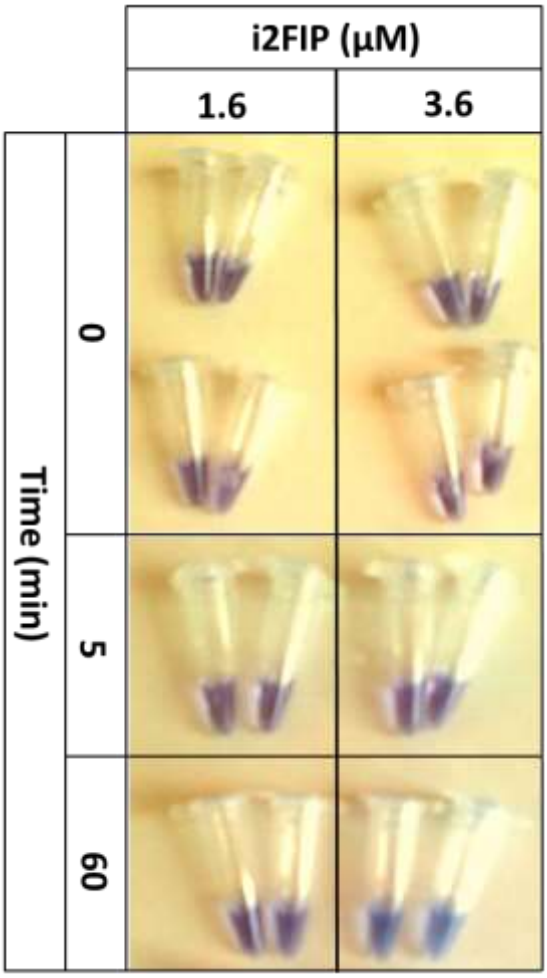


Figure 3.15 Effect of different amounts (µM) of i2FIP primer-template on HNB-induced colour progression for 5 minute and 60 minute LAMP reactions respectively. 0 min: Reaction colour at the start of the reaction. Reaction performed with 7mM $MgSO_4$.

As determined in section 3.3.1, where the effect of $MgSO_4$ concentration on HNB-induced colour change was investigated, optimal $MgSO_4$ concentration occurred within the range of 6 mM – 8 mM, and the lower the concentration, the earlier a colour change was expected. Therefore, the HNB interactions were also tested in the presence of 6 mM $MgSO_4$ (Fig. 3.17).

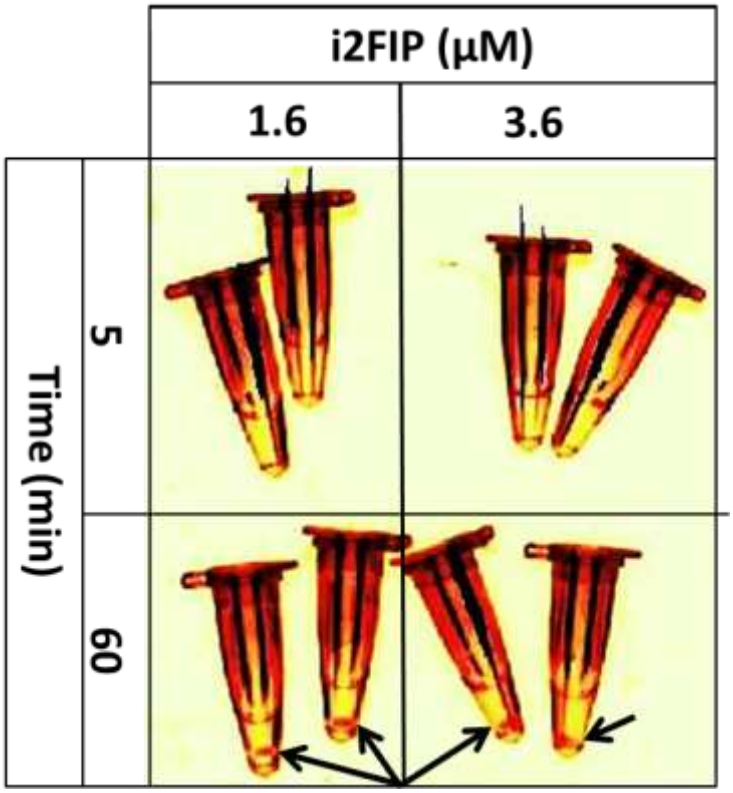


Figure 3.16 RT-LAMP reaction tubes viewed under white light to aid identification of the white $Mg_2P_2O_7$ amidst the blue or violet reaction mixture. The shaded areas indicated by arrows represent $Mg_2P_2O_7$ precipitate produced as the reaction progresses. No precipitate was present at the beginning of the reaction (5 min.).

The results were the same as that observed using 7 mM MgSO4 (Fig. 3.15), whereby amounts of 0-1.6 µM i2FIP primer did not change colour after 60 minutes and reactions using 3.6-16 µM i2FIP primer did change from violet to blue (Fig. 3.17). The lack of a colour change at the start of the reaction as well as 5 minutes into the reaction indicated that the colour change was solely the result of rate of increase in amplification (Fig. 3.17).

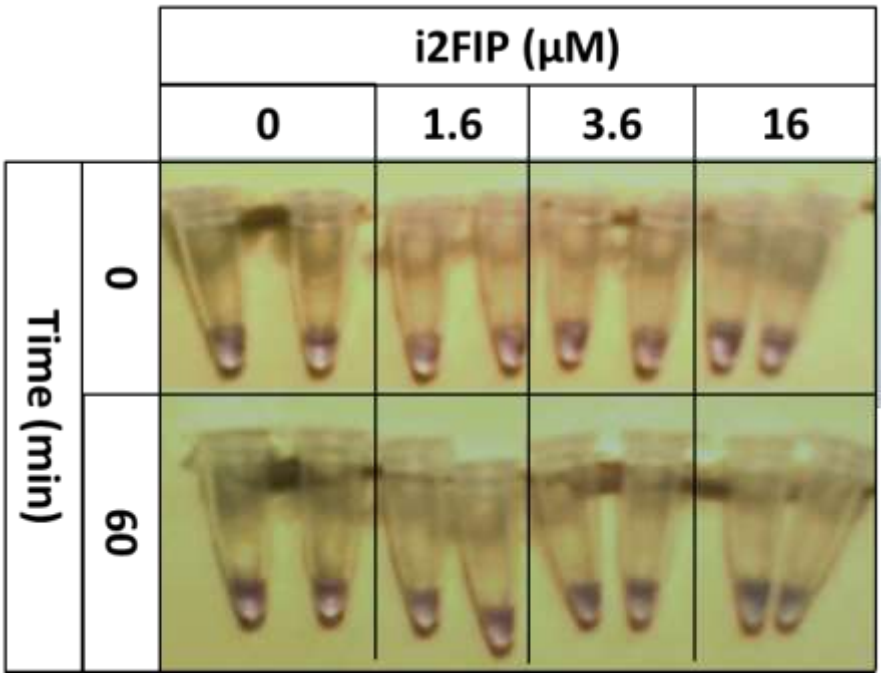


Figure 3.17 Effect of addition of different amounts (µM) of self-annealing forward inner primer-template (i2FIP) on HNB induced colour progression, using 6mM $MgSO_4$.

To further emphasise that the amount of FIP added affected the rate of amplification, agarose gel electrophoresis was performed using a DNA stain that produces increasing fluorescence with increasing amount of DNA present in the gel, this together with a corresponding set of mass standards (mass ruler) (Fig. 3.18). LAMP amplification product on the gel increased in brightness with increasing addition of template i2FIP primer, indicating an increase in mass (ng) for products of the same length (bp) (Fig. 3.18)

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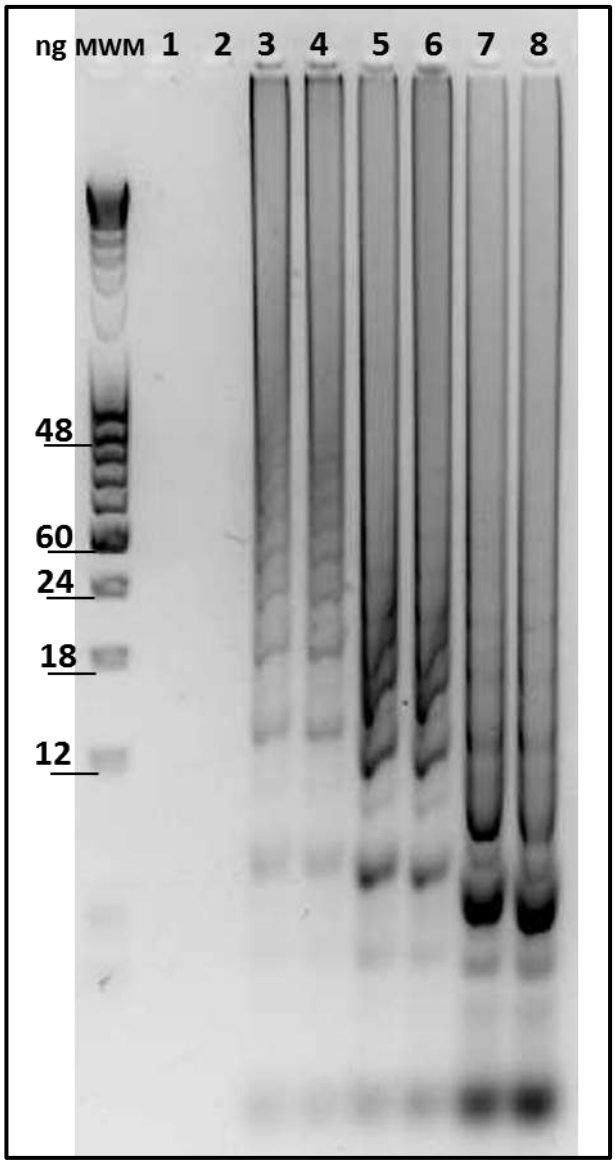


Figure 3.18 Amplification products as a result of RT-LAMP performed with increasing concentration of integrase-2 forward inner primer (i2FIP) primer-template. FIP concentration (µM): 0µM (Lanes 1-2); 1.6 µM (Lanes 3-4); 3.6 µM (Lanes 5-6) and 16 µM (Lanes 7-8). 2% TAE agarose gel stained with GR Green nucleic acid stain. MWM: 10Kb Mass Ruler™ DNA ladder (5 µL). ng: nanogram mass of reference ladder bands. Electrophoresis performed at 90V for 90 minutes. Lanes contain 5 µl of reaction product combined with 1 µl mass ruler loading dye.

3.3.4 Analysis of the colour change in response to HIV-1 amplification

RT-LAMP was performed using the integrase-DU179 primer set according to the conditions identified earlier (Section 3.3.1-3.3.3) for favourable HNB dye interactions. Specifically, the reaction was performed in the presence of 2U reverse transcriptase and 8 mM $MgSO_4$. After a 60 minute reaction at 60°C, the reaction was inactivated at 85°C for 5 minutes resulting in a pale blue reaction colour in the reaction containing HIV DU179

RNA (Fig. 3.19.1), whereas no colour change was observed in the no template control (Fig. 3.19.2) and RT negative controls (Fig.3.19.3) respectively.

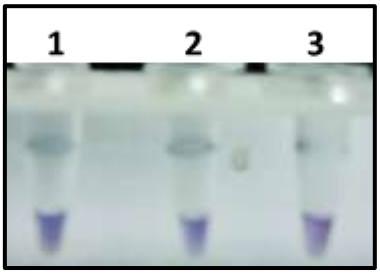


Figure 3.19 Reaction colour observed after performing RT-LAMP with integrase-DU179 primers at 60°C for 60 minutes. Reaction containing HIV DU179 RNA (tube 1), no template control (tube 2) and no RT control (tube 3).

Chapter 4

Discussion

Disease progression-monitoring in the HIV-infected population is a large contributor to successful treatment programmes. Various studies prove that HIV viral load is the most superior biomarker of identifying treatment failure and progression to AIDS and AIDS-related death (Mellors et al., 1997; Jourdain et al., 2013). However, the available commercial tests based on real time RT-PCR are expensive and complex. Viral load tests cannot be performed on-site at primary health care clinics. The complications of lengthy sample transport, the turn-around-time to result and the difficulty faced by patients in resource-limited settings to commit to follow-up emphasise the dire need for rapid, simple, on-site disease progression-monitoring, also referred to as point-of-care. Hence, the aim of this project was to develop a point-of-care test specifically by applying the novel reverse-transcription loop-mediated isothermal amplification technique, utilising hydroxy-naphthol blue colorimetric detection for semi-quantitative determination of HIV-1 viral load.

When compared to current low-cost strategies, such as RT activity assays and the LTR assay, RT-LAMP appeared most feasible as it has a rapid turn-around time and can be converted into a miniaturised, single step procedure, only requiring a heating block and centrifuge. Such advantages to the RT-LAMP assay have been applied in the production of non-commercial diagnostic assays for various infectious agents, but there is no HIV-1 viral load test existing in this format, and as such the foundational technology for an RT-LAMP assay needed to be developed.

Assay development is a multi-faceted procedure. As all aspects of the assay function in unison to achieve the purposed result, it was particularly challenging to systematically optimise individual reagents and conditions in isolation. Two essential areas where most of the effort was expended upon was in acquiring optimal viral template RNA; and then subsequently troubleshooting the somewhat complicated primer design. Despite these challenges and setbacks, a number of developments were achieved, and these are discussed below.

4.1. Template RNA acquisition

Various approaches were taken to obtain reasonable quality patient-sourced HIV RNA. RNA is easily degraded due to the abundance of RNA-nucleases in the environment. Since

the commercialisation of real time PCR viral load tests, numerous validations have been performed to determine how various plasma isolation and RNA extraction procedures affect the viral load result. We found that consistent quality was achieved by isolating plasma in serum separator tubes, followed by concentration of viral particles in 1ml of plasma by centrifugation. The resultant pellet is resuspended in PBS and applied to the Qiagen viral RNA procedure. These findings compared well with other studies. For example, PBS has been validated as an acceptable medium for altering sample input volume without compromising quantification (Mine et al., 2013). The use of gel-barrier tubes, such as serum separator and plasma separator tubes have been shown to improve RNA recovery (Ginocchio et al., 1997). The observed effects of plasma storage on RNA recovery agreed with the literature whereby samples are stored stably at -70°C (Ginocchio et al., 2007). As freeze-thaw cycles also affect stability (Ginocchio et al., 1997) plasma was therefore stored as 1 ml aliquots to avoid more than one freeze-thaw cycle.

4.2. Primer design and specificity to HIV-1

Key to the isothermal nature of RT-LAMP is the complex primer design whereby, following ~20 nucleotide portion of the inner primer hybridising to the target, the primer's 5' overhang loops back to bind to extending daughter DNA strand, catalysed by *BSt* polymerase (Notomi et al., 2000). Ultimately, primer design involves 4 primers corresponding to 6 regions of 1 target gene (Notomi et al., 2000), and an additional 2 primers that will hybridise to loop regions following initial amplification steps (Nagamine et al., 2002).

Although essential to the functionality of the technique, LAMP primer interactions are problematic and often prone to form off-target, self-primed amplicons (Gandelman et al., 2011; Curtis et al., 2009). It was therefore thought logical to apply a set of primer sequences which have already proved successful. A set of LAMP primers targeting the p24 region of the *Gag* gene, designed by Curtis et al. (2008) were tested for application in this viral load assay. The off-target amplification demonstrated here was similar to that observed by Curtis et al. (2009), which appeared to be products of primer homo- or hetero-dimerisation in the no template controls. However, they nevertheless demonstrated amplification of HIV RNA in the presence of both *in vitro* transcribed RNA and that obtained from patient samples. Yet no amplification of p24 RNA could be achieved in the

present study, wherein all electrophoretic bands appeared identical to the off-target amplification products, even though various sources of template were used.

Off-target amplification cannot be tolerated in a diagnostic assay, as it may lead to a false-positive result and therefore high levels of specificity are required. Hence, primer sets were tested not only in the presence of HIV RNA but alongside negative controls, such as no template controls (also referred to as water blanks) and reactions containing RNA obtained from HIV negative individuals or lambda phage DNA. This helped to ascertain off-target amplification produced by the first 3 primer sets as well as permit further testing with the fourth primer set, in which no template controls lacked any amplification.

The specificity of LAMP and RT-LAMP is considerably higher than conventional PCR as optimal binding of all six primers is required to bring about LAMP amplification of the desired target. Primer-target sequence identity of at least 90% is recommended (Zhang et al., 2010). In the context of HIV, however, this becomes problematic as the virus has no replication proof-reading capacity and is likely to mutate from progeny to progeny, the common cause of ART drug resistance. This may reduce the sensitivity of the RT-LAMP viral load assay, as certain strains of virus may not amplify, causing an underestimated viral load or false negative result (Wojewoda et al., 2013). The effect of genetic heterogeneity on sensitivity was reported by Curtis et al. (2008; 2009) whereby different amplification sensitivities were observed for detection of a homogenous viral load panel (as low as 12 copies HIV RNA) (Curtis et al., 2009) and patient samples (no less than 500 copies) (Curtis et al., 2008). High levels of genetically diverse viral strains can evolve in a single patient (Neher and Leitner., 2010; Lee et al., 2008), and may explain why, when using the p24 primers here, only off-target amplification occurred and no HIV was detected. This needn't be a drawback when using the assay in a semi-quantitative manner, where the desired detection limit is 1 000 copies. Still, the p24 primers failed to detect more than 1 000 copies of the WHO standard, a homogenous isolate, but this may have to do with the questionable quality of the RNA extracts.

Analysis of the RT-LAMP amplicons produced by the first 3 primer sets revealed identical electrophoresis patterns in negative controls and experimental reactions. This suggests that there was no binding to the template RNA or DNA by the first 3 primer sets. This was particularly unexpected with the use of the integrase-2 set in the presence of 2 integrase

clones. Although the clone sequences have 99% sequence identity with the integrase-2 primers (Appendix D), the subsequent amplification products were the same as those produced in the presence of lambda phage DNA or water.

Despite the strong sequence identity between the integrase clones and the integrase-2 primer set, binding to the target was prevented by self-priming within the forward-inner-primer of integrase-2 (i2FIP). This means only 5 of the 6 primers were available to bind to the target, which is not enough for LAMP amplification. Surprisingly, the i2FIP primer displayed higher self-affinity than affinity for the target sequence. This was evident as amplification did not occur in the absence of this single primer, but was present even in reactions from which all other primers and nucleic acid was excluded. These results were consistent whether in the presence of DNA or RNA, and whether reverse transcriptase was included or not.

Thus far it became clear that it was essential to stringently screen all primer sequences for any homo- or hetero-dimer sites before selecting for synthesis. This was done for the fourth primer set. Furthermore, to eliminate failure due to mismatches between the primers and target, the primers were designed specifically for the DU179 isolate, available as a tissue-culture supernatant. There are considerable differences between the use of culture supernatant and the testing of patient-derived RNA in the clinical setting. Culture supernatant is purer, lacking the proteins and molecules that are common in both healthy and diseased individuals that often complicate RNA extraction procedures. The culture-supernatant contains viral particles of a single isolate, here DU179 was used, consisting of very minimal genetic heterogeneity if any, whereas that of patients cannot be predicted. Originally, the most plausible strategy seemed to be to use patient-derived RNA so as to imitate the expected clinical pressures under which the assay would need to perform, but many problems were encountered with acquisition of RNA from patient plasma.

The difficulties with patient plasma-derived RNA included low yields(Table F1). Low yields of extracted RNA are common for cell-free fluids (Bustin et al., 2009). Variability in the Qiagen viral RNA kit has been reported previously (Fransen et al., 1998; Fanson, Osmack and Bisceglie, 2000; Monleau et al., 2009), yet in other cases the kit displayed superior reproducibility (Verhyden et al., 2003). RNA-dependent procedures are generally

fragile, the complexities of which do affect efficient, established routine testing (Aguilera et al., 2000).

Although part of the investigation required optimising acquisition of patient plasma-derived RNA, the aim was not to develop RNA extraction methodologies but rather RT-LAMP based HIV RNA quantification. The use therefore of the DU179 derived RNA and DU179 specific primers allowed for progress in the development of other aspects of the RT-LAMP assay that seemed hindered with the use of other sources of RNA (Appendix F). No homo- or hetero-dimer sites could be identified by the jPCR program for the DU179 primer set. As expected, this primer set successfully hybridised to the DU179 RNA, evident as the ladder-like banding pattern by subsequent gel electrophoresis. Product positivity was confirmed by target-specific restriction digestion. The presence of extra bands in the electrophoresis pattern following restriction digestion of LAMP products is thought to represent incomplete digestion due to the presence of multiple stems and loops (Le Roux et al., 2009).

4.3. RT-LAMP optimisation

A) Effect of magnesium and AMV RT on colour change

Following identification of a suitable set of primers for the assay, further development could take place. The general reaction conditions necessary for RT-LAMP amplification are well defined in the literature. Therefore, further optimisations only included conditions which specifically affected amplification detection by hydroxy-naphthol blue dye and the semi-quantitative nature thereof. Such parameters included optimising $MgSO_4$ concentration and reverse transcriptase. The findings are summarised in Table 4.1. In accordance with that observed by Goto et al. (2009) and Hadersdorfer et al. (2011), the colour change was found to be optimal at a final concentration of 6-8 mM $MgSO_4$. Since the colour change is related to free Mg^{2+} depletion, it is also affected by dNTP concentration (Zippelius et al., 2000; Goto et al., 2009). During extension of a DNA strand, Mg^{2+} forms complexes with the dNTP's (Zippelius et al., 2000). Since a longer template would use more dNTP's, the upper $MgSO_4$ limit of 8 mM was applied for the use of HIV amplification. While lower concentrations such as 6-7 mM favour an earlier colour change (Goto et al., 2009) and prevent off-target amplification (Liu et al., 2013a), concentrations below 8 mM can hinder amplification (Tsai et al., 2009). The lower limit of 6-7 mM

$MgSO_4$ was sufficient for the HNB interaction investigations where a 40 bp primer was applied as DNA template.

The RT effect may also be due to the use of Mg^{2+} and dNTP's during cDNA synthesis. Additionally, the RT effect may be specific to the type of RT used. It is generally recommended to perform RT-LAMP with 10 units of AMV RT, but this produced a colour change prior to the start of the reaction, which is a false-positive result. Instead, the amount of AMV RT was reduced to 2 units, wherein the reaction colour remained violet prior to incubation. The use of 2 units has previously proved to be sufficient for RT-LAMP amplification of HIV in the absence of HNB (Curtis et al., 2009). In another reported study, the use of 20 units of Superscript III RT did not produce a premature colour change (Hadersdorfer et al., 2011). Although not confirmed, the interaction between HNB and RT may be due to the presence of alkaline-earth metals in the RT carrier buffer.

B) RT-LAMP reaction temperature

Reaction temperature in nucleic acid testing requires stringent control. In PCR, an incorrect primer annealing temperature can cause off-target amplification or prevent target amplification. Essentially, RT-LAMP is performed optimally at the annealing temperature of the F3 and B3 primer sequences (Notomi et al., 2000). For this reason, some primer sets were subjected to RT LAMP incubation at a temperature as low as 55°C, though generally RT-LAMP is performed between 60°C and 65°C. In accordance with other LAMP applications (Liu et al., 2013a), the RT-LAMP reaction was found to be quite robust with regards to temperature across all 4 primer sets. Specifically, the first 3 sets, excluded due to off-target amplification, performed consistently at different incubation temperatures. The DU179 primers successfully amplified HIV at 59°C, 60°C and 62°C (Table 4.1). The robustness of the reaction makes it more flexible for field use applications where the heating source may fluctuate by a few degrees (Kubota et al., 2013) and will therefore also have fewer calibration demands than a thermocycler, for example.

Table 4.1 Optimum RT-LAMP reaction parameters for use with hydroxy-naphthol blue detection and primers targeting integrase.

Parameter	Optimum amount
$MgSO_4$	8 mM
AMV RT or Thermoscript RT	2 units
Incubation temperature	59°C, 60°C and 62°C

4.4. Mechanisms of hydroxy-naphthol blue amplification detection

The colorimetric result read-out of the proposed RT-LAMP assay is based on the interaction between hydroxy-naphthol blue dye and the RT-LAMP by-product, magnesium pyrophosphate. As dNTPs are added to the growing RT-LAMP products, the released phosphate group binds to free magnesium ions donated by magnesium sulphate, forming $Mg_2P_2O_7$. Therefore the by-product accumulates as the target nucleic acid is amplified. This produces turbidity in the reaction mixture, that when measured in real time is directly proportional to amplification rate (Mori et al., 2004).

The rate of PCR amplification is dependent on template amount. Similarly, it has also been demonstrated that LAMP amplification rate can be correlated with template quantity (Le Roux et al., 2009; Zhang et al., 2010). The coupling of a turbidometer to a LAMP reaction in real-time can therefore be used to determine viral load (Le Roux et al., 2009). Thus, as such, it was investigated here whether the amplification rate could then be correlated with the dynamics of the hydroxy-naphthol blue dye. In the absence of a source of known HIV template copy number, the previously identified self-annealing primer i2FIP was applied as a copy-number-controllable template. A definite correlation between initial i2FIP concentration and colour change from violet to blue was observed, supporting the suggestion that HNB can behave semi-quantitatively. This was also seen in qualitative, diagnostic applications of HNB detection of LAMP products, whereby assessing copy number sensitivity of these assays showed that reaction with higher amount of template displayed brighter blue colour change than that of lower copy numbers (Wang et al., 2012).This indicates that the application of the HNB dye to a semi-quantitative context was successful. Also the amount of i2FIP included in a conventional LAMP reaction (1.6 µM) did not produce a colour change after 60 minutes, indicating that self-primed amplification, common in LAMP reactions (Curtis et al., 2009) is unlikely to create a false-positive colour change by HNB. This suggests that HNB detection is more suitable than DNA intercalating dyes such as Sybr Green or Pico Green, which will detect any double-stranded DNA produced in a reaction.

The limitations of the use of i2FIP should however be considered: i2 FIP is a deoxyribo-oligomer only 40 nucleotides long. Specific use of the dye in the context of HIV RNA template averaging 470 nucleotides, and the associated reverse transcription dynamics, is

yet to be determined. However, Ma et al. (2010) observed a similar effect whereby HNB colour change took place at a threshold RT-LAMP detection of 60 copies H1N1 RNA, despite the presence of turbidity in violet reactions of lower copy number.

In the present study, once the HNB optimisations were applied to the HIV-DU179 sample, a colour change from violet to faint blue was observed after 60 minutes. This was a promising result as it suggests that the HNB-colour change dynamics may be applicable to the reverse transcription LAMP reaction, and not just in DNA amplification. However, the exact viral load of the culture supernatant was unknown but expected to be high, since tissue culture has been applied previously to permit viral replication under controlled conditions.

Further work was then required to determine the time-to-positive: the required RT-LAMP incubation time at which the colour change from violet to sky-blue is indicative of a HIV viral load $\geq$ 1 000 copies/ml. Current viral load tests provide absolute counts of viral copy number. However, this is not necessary for disease progression-monitoring, as seen in studies where by prediction of disease progression is achievable by placing patients within viral load ranges (Mellors et al., 1997). This basically implies that a patient having a viral load of 600 copies per ml has the same prognosis as a patient with a viral load of 800 copies per ml, as both fall in the category of above 500 copies. Similarly, two patients undergoing ART whose viral load changes from undetectable to 1 860 copies and undetectable to 900 respectively, will both be considered to have undergone treatment failure and would be managed accordingly. This provides more options in terms of designing resource-limited assays, and suggests that, though possibly limited in its inability to identify absolute viral load, the technique is still clinically relevant.

Currently, viral load control panels only exist in the form of sub-type B and would not be fitting for an assay developed for sub-type C. Instead, whole blood of 10 HIV-infected individuals was collected for the RT-LAMP assay and for routine viral load testing simultaneously. The routine viral load results were then used as a reference for further development of the assay, whereby the subsequently extracted RNA was diluted to obtain a range of copy numbers. Use of these samples, however, produced inconclusive results which appear to be due to the return of non-specific amplification withtin the reaction (Appendix F). It can also be questioned as to whether inhibition of an aspect of the RT-

LAMP reaction occurred as a result of co-extracted inhibitory molecules occurring in plasma. This is supported by the observation that use of patient-derived RNA proved problematic throughout the development of the assay Alternatively, a sub-type C viral load control panel would have to be synthesized by cloning of HIV-1 DNA and performing *in vitro* transcription to produce RNA. The advantage of this is that the synthesized RNA can be controlled for copy number and diluted to make up a viral load panel. The panel can then be used to perform RT-LAMP for a range of durations to determine the incubation duration for which panel members of ≥ 1 000 copies/ml could be distinguished by colour change from violet to blue. This should then be followed by validation on patient samples of unknown viral loads to determine the positive and negative predictive values of the RT-LAMP HNB viral load assay, compared with results obtained from simultaneous viral load real-time RT-PCR testing.

Chapter 5

5.1 Conclusion

This study represents the initial steps of assay development of a novel reverse-transcription loop-mediated isothermal amplification (RT-LAMP) viral load test, with a semi-quantitative, colorimetric result read-out. Aspects of assay development which were completed included the design of RT-LAMP primers against the integrase region of the *pol* gene, optimisation of reaction conditions including $MgSO_4$ concentration, reverse-transcriptase amount, reaction temperature, as well as the demonstration of the semi-quantitative capacity of the hydroxy-naphthol blue dye. The progress made towards development of the assay is summed up in the demonstration of the qualitative detection of the HIV-1 DU179, evident as a HNB-induced colour-change from violet to blue while the employed negative controls remained violet, following application of the optimisations that were carried out. The use of patient-derived RNA did not, however produce the same result and as such further considerations and optimisations are required to better suit the assay for use on patient samples.

5.2 Future work and considerations

RT-LAMP at the point-of-care

Due to the appeal of the LAMP and RT-LAMP formats to resource-limited settings, development of point of care heating-devices and lab-on-chip cartridge formats able to support the LAMP reaction are becoming more and more prevalent. The reagents can be lyophilised, easing the requirements for kit storage (LaBarre et al., 2011). Of particular interest is the development of non-instrumented, nucleic acid amplification (NINA) heating devices which rely on heat produced by an exothermic chemical reaction instead of electricity (LaBarre et al., 2011; Curtis et al., 2012; Kubota et al., 2013). NINA devices can achieve LAMP incubation temperatures with the use of boiling water in an ambient environment (Kubota et al., 2013). Similarly, there is a rise in sample collection development such as dried blood spots (Johannessen et al., 2009) and plasma separating devices that abrogate the need for a centrifuge (Liu et al., 2013b).

Recommendations for field use

RT-LAMP assays generally boast high specificity but this equates to low sensitivity in the context of the viral genetic heterogeneity characteristic of HIV-1, which could mean a poor positive-predictive value in some patients (Wojewoda et al., 2013). A simple means to

increase the positive-predictive value of the assay is to combine an undetectable result with other routine surrogate markers accessible at primary healthcare centres, such as haemoglobin and body mass index. An individual with notable changes in the surrogate markers but no corresponding RT-LAMP viral load change could be eligible for more sensitive, off-site tests including real time RT-PCR, CD4 count or even resistance screening in the case where a false-negative is suspected.

Chapter 6

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Appendix A: Human Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Denise Evans

CLEARANCE CERTIFICATE	**M10418**
PROJECT	Low-Cost Monitoring of HIV in Research-Limited Settings
INVESTIGATORS	Dr Denise Evans.
DEPARTMENT	Department of Medicine/Clinical HIV Res Unit
DATE CONSIDERED	30/04/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 11/06/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr D Evans

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix B: Materials

RNA extraction:

WHO 2nd International Standard for HIV-1 97/650 (NIBSC, Hertfordshire, UK) lyophilized human plasma reconstituted in SABAX dH_2O (Adcock Ingram, Midrand, South Africa) (1 ml), stored at -20°C, (50 µl aliquots).

Phosphate Buffered Saline (PBS) pH 7.4 (0.01 M) (Sigma-Aldrich, St Louis, USA) 1 tablet, dH_2O (200 ml) (0.0027 M potassium chloride, 0.137 M sodium chloride), stored at 4°C.

Carrier RNA (Qiagen, Hamburg Germany) (1 µg/µl) carrier RNA (310 µg), Buffer AVE™ (310 µl), aliquotted (12 µl), stored at -20°C.

Buffer AVE™ (Qiagen, Hamburg Germany) RNase-free dH_2O, 0.04% Sodium Azide.

Buffer AVL™ (Qiagen, Hamburg Germany) proprietary, contains guanidine thiocyanate.

Buffer AW1™ (Qiagen, Hamburg Germany) proprietary, contains guanidine hydrochloride, 100% EtOH (molecular grade) (Sigma-Aldrich, St Louis, USA).

Buffer AW2™ (Qiagen, Hamburg Germany)proprietary, contains sodium azide, 100% EtOH (molecular grade) (Sigma-Aldrich, St Louis, USA).

LAMP and RT-LAMP:

10X DNA polymerase Buffer B (Lucigen, Middleton, USA) Tris-HCl (20 mM), $(NH_4)_2SO_4$ (10mM), $MgSO_4$ (2mM) and 0.1% Triton X-100.

Hydroxy-naphthol blue (HNB)(3 mM) filter-sterilised (0.2 µm): HNB (SAS Chemical, Mumbai, India)(17.955 mg), 0.1% DEPC-treated dH_2O (10 ml).

DEPC-treated dH_2O (0.1%) autoclaved: Diethylpyrocarbonate (Sigma-Aldrich, St Louis, USA) (1 ml), dH_2O (1 L).

Restriction digestion:

10X *BSe*XI Buffer (Thermo Scientific, Massachusetts, USA) Tris-HCl (50 mM; pH 7.5), $MgCl_2$ (2 mM), NaCl (100 mM), BSA (0.1 mg/mL).

Agarose gel electrophoresis:

Tris – Boric Acid – EDTA Buffer (TBE) (5X) TrisHydroxy-methyl aminomethane (27 g), Boric Acid (13.75 g), EDTA (2.325 g), distilled water (500 ml).

1X TBE 5X TBE (100 ml), distilled water (400 ml).

Tris – acetic acid – EDTA Buffer (TAE) pH 7.6 (10X) Tris Hydroxy-methyl aminomethane (96.88 g), Sodium acetate.$3H_2O$ (13.6 g), EDTA (7.44 g), dH_2O (2 L).

1X TAE 10X TAE (100 ml), dH_2O (900 ml).

1kb GeneRuler working solution 1Kb GeneRuler (Thermo Scientific, Massachusetts, USA) (1 µl), 6X Loading dye (Thermo Scientific, Massachusetts, USA) (2 µl), nuclease-free distilled water (Thermo Scientific, Massachusetts, USA) (9 µl).

6X DNA Loading dye (Thermo Scientific, Massachusetts, USA) 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA

2% Agarose gel Agarose (Lonza) (1.2 g), 1X TBE/TAE (60 ml), GR Green Nucleic Acid gel stain (Clare Chemical) (6 µl).

1.2% Agarose gel Agarose (Lonza) (0.72 g), 1X TBE/TAE (60 ml), GR Green Nucleic Acid gel stain (Clare Chemical) (6 µl).

Appendix C: Primer sequences

Table C1. The 6 primers sequences of each of the 4 RT-LAMP primer sets used for the development of the RT-LAMP-based viral load assay. Italics indicate linkers recommended by Notomi et al. (2000)

Target gene	Primer sequence (5'-3')
p24 (*Gag)* (Curtis et al., 2008) (Inqaba Biotechnologies, Pretoria, South Africa)	FIP CAGCTTCCTCATTGATGGTTTCT*TTTT*AACACCATGCTAAACACAGT BIP TGTTGCACCAGGCCAGATAA*TTTT*GTACTGGTAGTTCCTGCTATG F3 ATTATCAGAAGGAGCCACC B3 CATCCTATTTGTTCCTGAAGG Loop F TTTAACATTTGCATGGCTGCTTGAT Loop B GAGATCCAAGGGGAAGTCA
Integrase-1 (University of Cape Town, Cape Town, South Africa)	FIP TTCTTTAATTCTTTATTCAT*TTTT*CAGGTATCCAACAGGAATTT BIP ACATACAAACTAAAGAACTATT*TTTT*TCCAAATAGGGTCTCTGC F3 TTTCACCAGTGCTGCAGTTAAGGCA B3 ACTACTGCCCCTTCACCTTTCCA Loop F TACTACTCCCTGACTTTGGG Loop B AATTCAAAATTTTCGGGTTTATT
Integrase-2 (Inqaba Biotechnologies, Pretoria, South Africa)	FIP TCTTTATTCATGGATTCT*AAT*TACATACAGACAATGGCAGT BIPAAAAGGGGGGATTGGGGGGT*AAAA*GGGTCTCTGCTGTCTC F3 ACTAAAATTAGCAGGAAGATGGC B3TTATGTCACTGTTATCTTGTATTACT Loop FTGGGGATTGTAGGGAAT Loop BCAGGGGGGAAAGAATAATAGATA
Integrase/DU179 (University of Cape Town, Cape Town, South Africa)	FIP GATACCTGCCCACCAACAGG*TTTT*AGACAATGGTAGTAATTTCAC BIPAATTCCCTACAATCCCCAAAGTC*TTTT*TCTTACCTGTCCTATGATT F3ACTAAAATTAGCAGGAAGATGGC B3 TGGAAAGGTGAAGGGGCAGTAGT Loop F GCCTTAACTCTAGCACTGG Loop B GGAGTAGTAGAATCCATGAATAAAGAATT

Appendix D: Integrase clone sequence identity to integrase-2 primers

Regions of primer hybridisation are underlined and mismatches highlighted in grey.

>FV20
TTTCTAGATGGAATAGATAAGGCTCAAGAAGAGCATGAAAAATATCATAGCAATTGG
AGAGCAATGGCTAGTGAGTTTAATCTGCCACCCATAGTAGCAAAAGAAATAGTAGCTA
GCTGTGATAAATGTCAATTAAAAGGGGAAGCCACACATGGACAAGTAGACTGTAGCC
CAGGGATATGGCAATTAGACTGTACACATTTAGAAGGAAAAATCATCCTGGTAGCAGT
CCATGTAGCCAGTGGCTACATAGAAGCAGAGGTTATCCCAGCAGAAACAGGACAAGA
AACAGCATACTATATACTAAAGGTTAGCAGCAAGATGGCCAGTCAAAATAATACATACA
GACAATGGCAGCAATTTCACCAGTGCTACAGTTAAGGCAGCCTGTTGGTGGGCAGGTA
TCCAACAGGAATTTGGAATTCCCTACAATCCCCAAAGTCAGGGAGTGGTAGAATCTAT
GAATAAAGAATTAAAGAAAATCATAGCACAAGTAAGGGATCAAGCTGAACATCTTAA
GACAGCAGTACAAATGGCAGTATTCATTCACAATTTTAAAAGAAAAGGGGGGATTGG
AGGGTACAGTGCAGGGGAGAGAATAATAGACATAATAGCAACAGACATACAAACTAA
AGAATTACAAAAACAAATTTTAAAAATTCAAAATTTTCGGGTTTATTACAGAGACAGC
AGAGACCCTATTTGGAAAGGACCAGCCAAACTACTCTGGAAAGGTGAAGGGGCAGTA
GTAATACAAGATAATAGTGACATAAAGGTAGTACCAAGGAGAAAAGCAAAAATTATT
AGGGACTATGGAAAACAGATGGCAGGTGCTGATTGTGTGGCAGGTAGACAGGATGAG
GATCAGAACATGGAATAG

Total mismatches: 8 (0.91%) = 99% match

>FV23
TTTCTAGATGGAATAGACAAGGCTCAAGAAGAGCATGAAAAATACCACAGCAATTGG
AGAGCAATGGCTAATGAGTTTAATCTGCCACCTGTAGTAGCAAAAGAAATAGTAGCTA
GCTGTGATAAATGTCAGTTAAAAGGGGAAGCCATGCATGGACAAGTAGACTGTAGTCC
AGGGATATGGCAATTAGACTGTACACATTTAGAAGGAAAAACCATCTTAGTAGCAGTC
CATGTAGCCAGTGGTTACATAGAAGCAGAGGTTATCCCAGCAGAAACAGGACAGGAA
ACAGCATACTATATACTAAAGCTAGCAGGAAGATGGCCAGTCAAAGTAATACATACA
GACAATGGCAGTAATTTCACCAGTACTGCAGTTAAGGCAGCCTGTTGGTGGGCAGGCA
TCCAACAAGAATTTGGAATTCCCTACAATCCTCAAAGTCAGGGAGTAGTAGAATCCAT
GAATAAAGAACTAAAGAAAATTATAGGGCAGGTAAGAGATCAAGCTGAGCACCTTAA
GACAGCAGTACAAATGGCAGTATTCATTCACAATTTTAAAAGAAAAGGGGGGATTGG
GGGGTACAGTGCAGGGGAAAGAATAATAGACATAATAGCAACAGACATACAAACTAA
AGAACTACAAAAACAAATTATAAAAATTCAAAATTTTCGGGTTTATTACAGAGACAGC
AGAGACCCTATTTGGAAAGGACCAGCCAAACTACTCTGGAAAGGTGAAGGGGCAGTA
GTGATACAAGATAATAGTGACATAAAGGTAGTACCAAGAAGGAAAGCAAAAATTATT
AGGGACTATGGAAAACAGATGGCAGGTGCTGATTGTGTGGCAAGTAGACAGGATGAG
GATCAGAACATGGAATAG

Total mismatches: 6 (0.68%) = 99% match

Appendix E: Additional protocols

RT-LAMP product purification by GeneJet™ PCR purification kit (Thermo Scientific, Massachusetts, USA)

- 1:1 volume of binding buffer (proprietary) was added to LAMP reaction mixture, vortexed for 10 seconds, spun down for 10 seconds
- The solution was applied to the centre of a GeneJet™ purification column and centrifuged at 12000xg for 1 minute.
- Flow-through was discarded and wash buffer (700 µl) (proprietary) added to the column and centrifuged at 12000xg for 1 minute.
- Flow-through was discarded and the column centrifuged dry at 12000xg for 1 minute.
- Column was placed in a sterile 1.5 ml eppendorf tube and elution buffer (10 mM Tris-HCl) (30 µl) was applied to the column and incubated at ambient temperature for 1 minute.
- The column was centrifuged at 12000xg for 1 minute and the eluate quantified by Nanodrop™ Spectrophotometry.

Appendix F: Optimised RT-LAMP performed on serial dilutions of patient-derived plasma of known viral load concentrations

In following the optimised RNA extraction procedure (section 3.1), a subset of 10 patient samples of known viral loads were collected for later use to aid the determination of the time point in the reaction at which a colour change from violet to blue indicates a viral load ≥ 1000 copies HIV RNA/ml. Following RNA extraction, dilutions of copy numbers were tested with the optimised RT-LAMP assay (Fig. F1), however this resulted in non-specific amplification (Fig. F1.B, lanes 6-8) and no colour change from violet to blue was produced (Figure F1.A).

Table F1 Comparison of HIV-1 viral load and concentration of RNA extracted using the Qiagen Viral RNA extraction procedure following SST purification and viral particle concentration of 1ml of HIV patient-derived plasma. Concentration determined by Nanodrop™ spectrophotometry. Viral load was determined by real-time RT PCR-based routine testing at a centralised viral load laboratory.

Sample ID	C (ng/µl)	Viral Load (copies RNA/ml)
H16	83.5	245 395
H13	150.5	140733
H17	113.7	120 950
H12	25.7	62774
H14	135.1	14195
H18	89.46	5810

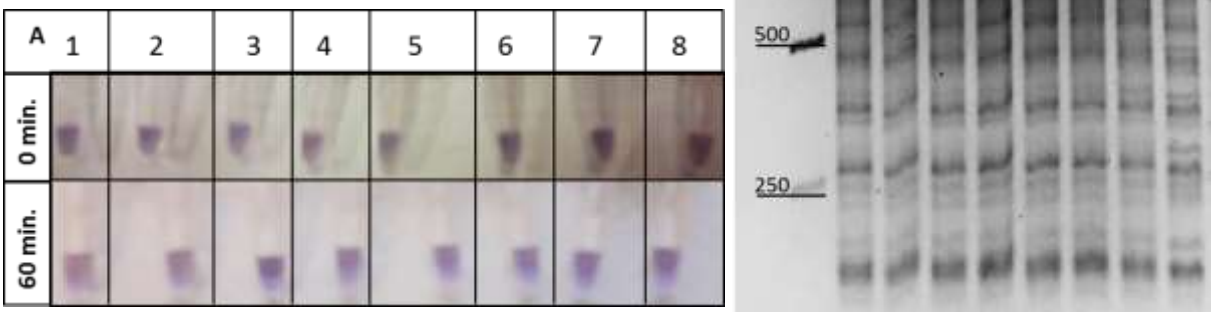


Figure F1 RT-LAMP performed on dilutions of HIV-infected plasma-derived RNA to assess sensitivity of the viral load assay to distinguish varying viral loads. A) Reaction colour at start (0 min.) and end (60 min.). B) RT-LAMP reaction products analysed by 2% T.B.E. agarose gel electrophoresis. Viral load of the plasma samples was determined by real-time RT-PCR at a routine viral load laboratory on replicate plasma samples. The range of HIV copy numbers tested included 370 000 (1 and 6), 5000 (2), 1000 (3 and 4) and 500 (5 and 7) alongside RT-negative (6 and 7) and no template controls (8). MWM: 1Kb DNA ladder.