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Cost Effective Diagnosis and Monitoring of HIV-1 in a Resource Poor Setting

Natela Rekhviashvili

A thesis submitted to the Faculty of Health Sciences,

University of the Witwatersrand, in fulfillment of the requirements for the

degree of Doctor of Philosophy

Johannesburg, 2007

DECLARATION

I, Natela Rekhviashvili, declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

……....day of……, 2007

Dedicated to my parents,

Prof. I. Rekhviashvili and Mrs. E. Rekhviashvili

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Publications in Internationally accredited journals

Rekhviashvili, N., Stevens, W., Marinda, E., Gonin, R., Stevens, G., McIntyre, J. and Wood, R. (2006) Clinical performance of an in-house real-time RT-PCR assay using a fluorogenic LUX primer for quantification of Human Immunodeficiency Virus type -1 (HIV-1). (J Virol Methods. In press).

Rekhviashvili, N., Stevens, G., Scott, L. and Stevens, W. (2006) Fluorogenic LUX primer for quantitation of HIV-1 by real-time RT-PCR. Molecular Biotechnology 32, 101-110.

Stevens, G., **Rekhviashvili, N**., Scott, L., E., Gonin, R., and Stevens, W. (2005) Evaluation of two commercially available, inexpensive alternative assays used for assessing viral load in a cohort of human immunodeficiency virus type 1 subtype C-infected patients from South Africa. J Clin Microbiol 43(2), 857-861.

Patent Applications

International PCT/Patent application filed for the method for determining HIV-1 viral load. Reference Number: PCT/IB2006/000182; 1 February 2006.

Inventors: **Rekhviashvili, N**., Stevens, G., Stevens, W.

(Spoor & Fisher, Patent, trade mark and copyright attorneys)

South African provisional patent application (number 2006/10433) filed for a LMP (Loop Mediating Primer) design and the RT-LDA (Reverse Transcription Loop Dependant

Amplification) technique. Filed on 13th December 2006.

Inventors: **Rekhviashvili, N**., Stevens, W.

(Spoor & Fisher, Patent, trade mark and copyright attorneys)

Conference presentations

Rekhviashvili, N. and Stevens, W. Oral presentation on: Approaches to affordable HIV viral load monitoring. 2nd Symposium Session: Low cost diagnostic monitoring for scaling up treatment programmes. South African AIDS Conference, Durban, RSA. 7-10 June 2005.

Rekhviashvili, N., Stevens, G., Galpin, J. and Stevens, W.. Development of an affordable HIV-1 viral load assay for reference laboratories in resource constrained settings. 11th CROI (Conference on retroviruses and opportunistic infection), San-Francisco, CA, USA. 8-11 February 2004, abstract number 590.

Rekhviashvili, N., Stevens, G., Corrigan, G. and Stevens, W. Reverse Transcriptase assay (Cavidi ExaVir™) as a cost effective alternative HIV-1 viral load for Africa. 2nd IAS (International AIDS Sociaty) conference. Paris, France. 13-16 July 2003, abstract number 476.

Stevens, G., **Rekhviashvili, N.,** Scott, L. and Stevens, W. Affordable HIV-1 monitoring: a direct comparison of an improved p24 antigen assay and the Roche Amplicor Monitor assay. 2nd IAS (International AIDS Sociaty) conference. Paris, France. 13-16 July 2003, abstract number 475.

ABSTRACT

The South African National Antiretroviral Treatment Guidelines recommend the use of HIV-1 viral load assays for routine monitoring of HIV-1 positive patients receiving highly active antiretroviral therapy (HAART). This thesis describes the innovative approaches to developing more affordable HIV-1 diagnostics and monitoring assays for South Africa, which take into account the tiered laboratory infrastructure of this country.

An in-house HIV-1 viral load assay – the LUX assay, was developed and evaluated with a view of implementing this more affordable option in high tier laboratories. The LUX assay represents quantitative real-time RT-PCR that utilizes the LightCycler® technology (Roche) in a novel combination with a LUX™ primer. The assay showed good analytical sensitivity, specificity and reproducibility of its linear dynamic range of $4x10^2$ to $4x10^6$ RNA copies/ml. Preliminary clinical evaluation (n = 458) of the LUX assay showed good agreement with the COBAS Amplicor assay, and demonstrated its usefulness for long term monitoring of HAART patients.

ELISA based viral load testing approaches were investigated as low cost and less technically complex alternatives for medium tier laboratories. The HiSens HIV-1 p24 Ag Ultra (Perkin Elmer) and the ExaVir™ Load Quantitative HIV-RT kits (CAVIDI) were compared with the Roche Amplicor assay. Both assays showed strong association with the Roche Amplicor assay, with $R^2 = 0.686$ and $R^2 = 0.810$, respectively (n = 117). These alternative assays seemed most useful in the serial monitoring of patients on HAART. Major drawbacks included the wide variability of both assays, insufficient sensitivity of the p24 antigen assay and low throughput of the RT assay.

Development of a point-of-care HIV-1 RNA assay could address issues related to early and cost effective diagnosis of acute HIV infection. A novel isothermal amplification technique termed the Reverse Transcription Loop Dependant Amplification (RT-LDA) was developed as one component for a potential point-of-care HIV-1 RNA assay. The RT-LDA converted RNA into partially looped ssDNA amplicons, over a wide RNA concentration range ($4x10^3$ to $4x10^8$ copies/ml) using a 1 hour incubation at 53ºC. The RT-LDA technology is fully compatible with a lateral flow detection system using dipsticks and highly suitable for point-of-care testing.

Overall, this study demonstrates the feasibility of developing novel, more affordable HIV-1 testing options that would be appropriate for the tiered laboratory infrastructure present in South Africa. Evaluation of commercially available, less expensive alternative HIV viral load assays in local settings facilitates their implementation.

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NOMENCLATURE

Ab – Antibody

AcBSA - Acetylated Bovine Serum Albumin

Ag - Antigen

AHI - Acute HIV infection

AIDS - Acquired Immune Deficiency Syndrome

AMV - Avian Myeloblastosis Virus

AP - Alkaline Phosphatase

ART - Antiretroviral Treatment

ARV - Antiretroviral

BBI - British Biocell International

BBI - Boston Biochemica Inc.

bp - Base Pairs

BrdUTP - Bromo-deoxyuridine triphosphate

bDNA - Branched DNA

ºC - Degree Celsius

CDC – Centre for Disease Control (USA)

cDNA - Complementary DNA

CI - Confidence Interval

CPT - Cycling Probe Technology

CTL - Cytotoxic T lymphocytes

CV - Coefficient of Variation

dATP - Deoxyadenosine triphosphate

dCTP - Deoxycytidine triphosphate

dGTP - Deoxyguanosine triphosphate

dH_2O - Distilled water

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic Acid

DNP - Dinitrophenyl

dNTP - Deoxynucleotide

dsDNA - Double Stranded DNA

dTTP - Deoxythymidine triphosphate

EDTA - Ethylenediaminetetra-acetic Acid

EIA - Enzyme Immunosorbent Assay

ELISA -Enzyme Linked Immunosorbent Assay

ES - External Standard

FAM - (6 or 5) – Carboxyfluorescein

FDA - Food and Drug Administration (USA)

fg - Femtograms

FRET - Fluorescence Resonance Energy Transfer

gag - Group Antigen

gp160 - full length envelope glycoprotein

gp120 - extracellular component of gp160

gp41 - transmembrane component of gp160

HAART - Highly Active Antiretroviral Therapy

HBV - Hepatitis B Virus

HCV - Hepatitis C Virus

HDA - Helicase-Dependant Amplification

HIV-1 - Human Immunodeficiency Virus Type 1

HL - UNG - Heat Labile Uracil - DNA – Glycosylase

IC - Internal Control

ICC - Intra-class Correlation Coefficient

Ig - Immunoglobulin

IN - Integrase enzyme

IPTG - Isopropylthiol- β - D – galactoside

IU - International Units

JOE - 6- carboxyl - 4', 5' - dichloro- 2', 7' – dimethoxyfluorescein

kb - Kilobases

kDa - Kilodalton

LAMP - Loop Mediated Amplification

LB - Luria-Bertani medium

LED - Light Emitting Diode

LIA - Line Immunoassay

LLQ - Lower Limit of Quantitation

LMP - Loop Mediating Primer

LUX - Light upon Extension

M - Molar

MAR - Magnetic Assay Reader

MGW - Molecular Grade Water

µg - Microgram

min - Minutes

ml - Milliliter

µl - Microliter

mM - Millimolar

NAAT - Nucleic Acid Amplification Technology

NALF - Nucleic Acid Lateral Flow

NASBA - Nucleic Acid Sequence Based Amplification

NAT - Nucleic Acid Tests/Testing

NHLS - National Health Laboratory Service

NHP - Negative Human Plasma

NIH - National Institute of Health (USA)

NTC - No Template Control

p24 antigen - Capsid core protein of HIV

PAGE - Polyacrylamide Gel Electrophoresis

PCR - Polymerase Chain Reaction

pH - Measurement of hydrogen ion concentration

pmol - Picomole

POC - Point - of - Care

pol - Polymerase gene of HIV

QC - Quality Control

RCA - Rolling Circle Amplification

RNA - Ribonucleic Acid

RNase H - Ribonuclease H

rpm - Revolutions per minute

RT - Reverse Transcriptase (enzyme)

RT - Reverse Transcription

RT-LDA - Reverse Transcription Loop Dependant Amplification

RT-PCR - Reverse Transcription Polymerase Chain Reaction

s - Seconds

SA - South Africa

SD - Standard Deviation

SDA - Strand Displacement Amplification

S-HRP - Streptavidine Horse Radish Peroxidase

ssDNA - Single stranded DNA

TAE - Tris-Acetate EDTA

TBE - Tris Boric acid EDTA

TEMED - N,N,N',N', - tetramethylethylenediamine

TMA - Transcription Mediated Amplification

UV - Ultraviolet

V - Volts

v/v - Volume per volume

VCT - Voluntary Counseling and Testing

VQA - Virology Quality Assurance

WB – Western Blot

WHO - World Health Organization

WITS - University of the Witwatersrand (SA)

X - gal - 5- Bromo - 4 - chloro- -3- -indolyl - β- D galactoside

CHAPTER 1

BACKGROUND

1.1 Rationale for the development and evaluation of affordable Human Immunodeficiency Virus Type 1 (HIV-1) diagnostic and monitoring assays in South Africa

The World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that an average of 39.5 million (34.1 - 47.1 million) individuals worldwide were infected with HIV by the end of 2006. This staggering number includes ~ 4.3 million (3.6 - 6.6 million) adults and children newly infected in 2006 and 2.9 million (2.5 - 3.5 million) people that died of AIDS related illnesses in the same year (UNAIDS). South Africa has one of the worst AIDS epidemics in the world currently showing no evidence of a decline. An estimated 5.5 million (4.9 - 6.1 million) people were living with HIV in South Africa in 2005 (UNAIDS).

According to the South African government's announcement made in April 2004, all HIV positive individuals classified as having stage 4 AIDS (WHO, 2005), or CD4 counts <200 cells/µl become eligible for anti-retroviral treatment (ARV). Currently, approximately 140 000 people in South Africa are enrolled on highly active anti-retroviral treatment (HAART) in the public health sector and 80 000 people in the private sector (MacFarlane, 2006). More than R3.4 billion has been allocated for procurement of antiretroviral drugs for the current period ending 2007 (Implementation of the comprehensive plan on

prevention treatment and care of HIV and AIDS; www.info.gov.za). Recent statistics reflected an increase in people tested at public health care facilities for HIV infection from 511 843 in 2003/4 to 1 019 476 during 2004/5 (Implementation of the comprehensive plan on prevention treatment and care of HIV and AIDS; www.info.gov.za). Implementation of Highly Active Antiretroviral Therapy (HAART) under the national roll-out program in South Africa raised a need for increased access to affordable laboratory tests for diagnosis and monitoring of therapy. Despite recent substantial decreases in the cost of ARV drugs, expensive laboratory tests still add significantly to the overall cost of an individual patient's treatment (Glencross et al., 2003). In the context of the incredibly large number of individuals requiring therapy in South Africa, scaling up laboratory investigations for monitoring treatment efficacy represents a significant challenge for the country. According to the South African National Antiretroviral Treatment Guidelines (National Department of Health, SA, 2004) routine monitoring of patients on HAART has to include determination of $CD4^+$ counts, HIV-1 viral load and alanine transaminase (ALT) testing. The recent development and implementation of a cost effective Pan*Leuco*gating (PLG) strategy for $CD4^+$ T-cell enumeration (Glencross et al., 2002) allowed for approximately 30% cost savings for the public sector ARV program in South Africa. Similar approaches have been investigated internationally and nationally in an attempt to reduce the cost of HIV viral load testing in resource constrained settings. Commercial tests that are currently used in South Africa for HIV-1 viral load determination remain expensive: the COBAS Amplicor HIV-1 Monitor™ 1.5 test (Roche Molecular Systems) and the NucliSens™ EasyQ (Biomérieux) cost ~ 45$ and ~75$ per test, respectively. Development of a more affordable in-house HIV-1 viral load assay will ease the financial burden of treatment and monitoring of AIDS patients in our country.

The realistic picture of South Africa's HIV/AIDS problem would not be complete without mentioning the difficulties of diagnosing incident cases that are HIV antibody negative by rapid or ELISA assay (Stevens et al., submitted). The importance of diagnosing HIV-1 infection in the acute phase is discussed in section 1.3 of this chapter. An overview of diagnostic tests (section 1.3) shows that currently not many technical approaches for the diagnosis of acute HIV-1 infection are available. In particular, HIV-1 p24 antigen detection and HIV-1 RNA quantitation (viral load) assays are the options for diagnosis of the acute phase of the infection. Both types of assays require a specialized laboratory set-up. To our knowledge, a rapid, RNA based "near patient" type of assay for diagnosis of acute HIV-1 phase is not commercially available. Figure 1.5 shows that viral RNA can be detected in plasma from week 1 post-infection, which makes nucleic acid testing (NAT) the methodology of choice for developing such a diagnostic test. In the South African context another difficulty of diagnosing acute infection is related to the loss to follow-up when patients do not return at a later date to receive their results. Therefore, the most desirable approach for our country would be a NAT based point-of-care (POC) test ensuring immediate post-test counseling at the voluntary counseling and testing (VCT) centers or primary health care clinics in the remote areas.

Public sector laboratory services in South Africa are provided by the National Health Laboratory Service (NHLS). The laboratory infrastructure of the NHLS is comprised of three laboratory tiers differing in their size, staffing resources, and the scope and complexity of the work undertaken. These are classified as follows: a) tertiary/reference/high tier laboratories located at the teaching hospitals; b) medium tier laboratories at district hospitals and c) primary health care laboratories (low tier) at subdistrict hospitals or clinic facilities (Bates and Mendelow, 2006). Generally, tertiary

care laboratories are capable of implementing “high tech” assays and handling large volumes of work. These facilities have the appropriate technical resources including constant electricity and water supply, freezer capacity and dedicated laboratory space for different procedures. Skilled personnel, receiving offices with trained phlebotomists, training programs and basic quality control (QC) procedures are also in place. Medium tier laboratories do not have these specialized facilities (except for water and electricity supply) and thus suitable assays for these environments are generally less complex techniques that provide medium or low throughput. Primary health care laboratories or clinic facilities can cope with conducting technically non-demanding assays such are rapid and POC tests. Laboratory facilities in South Africa are unevenly distributed throughout the country. High volume, reference laboratories constitute approximately 21% of all the laboratories in the public sector and they are located mainly at the large industrial, urban centers. Medium tier laboratories are affiliated to the secondary, district hospitals that are found in the urban and peri-urban areas and form ~10.2% of the total network. Primary health care laboratories are affiliated to primary health care clinics that are mostly located in the rural areas and they constitute ~ 68.8% of the total NHLS laboratory facilities in South Africa. Figure 1.1 shows the NHLS laboratory network in South Africa.

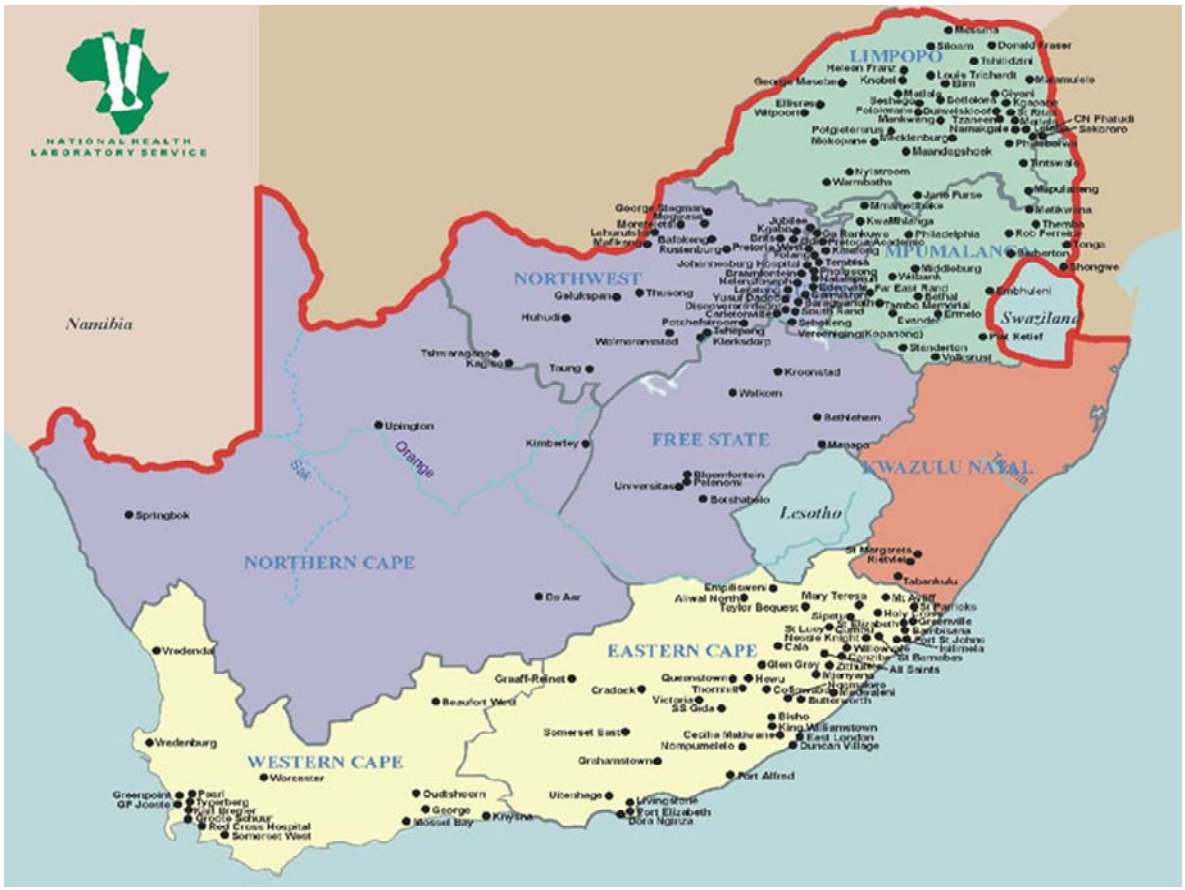


Figure 1.1: Distribution of NHLS laboratories in South Africa. The locations of NHLS laboratories on the map are indicated by the dots.

The overall aim of this study is to address South Africa's current problem related to the prohibitive cost of current HIV viral load assays for monitoring HIV infected patients on HAART, and absence of POC testing for diagnosing acute/primary HIV-1 infection. The study was designed taking into account different needs of different laboratory tiers in South Africa. The primary objectives of this study are:

- To develop and evaluate a more affordable, NAT based in-house HIV-1 viral load assay for implementation in high tier laboratories;
- To evaluate commercially available non-NAT based, cost effective alternative HIV-1 viral load tests suitable for medium tier laboratories in South Africa;

- To develop a novel isothermal amplification technique that can be used as part of an automated POC assay for detection of viral RNA during acute HIV-1 infection suitable for low tier laboratories in South Africa.

The structure of this thesis reflects the three primary objectives of this study. Chapter 2 is dedicated to the development and preliminary clinical evaluation of a new, more affordable HIV-1 viral load assay for monitoring the efficacy of HAART. The assay utilizes RNA quantitation based on real-time RT-PCR technology using the LightCycler® platform (Roche Applied Science, Mannheim, Germany) and was developed and evaluated using plasma samples from South African patients on HAART, with a view to implement testing in a reference, high volume laboratory. The main challenge of this project was to develop an HIV-1 viral load assay with performance characteristics compatible to those of current "gold standard" commercial assays, yet making it more affordable.

Chapter 3 describes an evaluation of two commercially available alternative assays for HIV-1 viral load monitoring - the Perkin-Elmer HD HiSens p24 assay and the Cavidi ExaVir® Load Quantitative HIV-RT assay. These assays are less technically demanding, require minimal capital outlay for the initial implementation and low maintenance and thus represent a potential viral load testing alternative for the medium tier laboratory level. The HiSens p24 Ag and the ExaVir™ HIV-RT assays were evaluated using plasma samples obtained from South African patients currently receiving ARV therapy.

Chapter 4 is related to the development of a novel isothermal RNA amplification technique for use in a NAT based POC assay for early diagnosis of acute HIV-1 infection. An amplification reaction (Reverse transcription Loop Dependent Amplification, RT-LDA) that could quickly and efficiently convert isolated viral RNA molecules into the single

stranded DNA (ssDNA) amplicons was developed. The proof-of-concept study for the development of RT-LDA was performed using a synthetic RNA target. Amplicons (ssDNA) were detected (in the visual range) by nucleic acid lateral flow (NALF) technology that makes use of nitrocellulose dipsticks. The use of a more sensitive detection format using a magnetic assay reader (MAR, Quantum Design, San-Diego, CA, USA) as well as future automation of the assay for POC testing is discussed.

The concluding chapter (chapter 5) summarizes the most important findings and conclusions of this study. This chapter also discusses future directions of research and development in the field of HIV-1 diagnostics and monitoring in resource poor settings.

1.2 Commercial assays available for HIV-1 viral load quantification

Plasma viremia during the course of HIV-1 infection is commonly referred to as plasma viral load. HIV-1 viral load is measured by quantifying the number of viral RNA copies per milliliter (ml) of plasma. Before seroconversion extremely high levels of plasma viremia are maintained by rapid viral replication, which in turn causes continuous re-infection and destruction of $CD4^+$ T cells. Due to the onset of host immune responses the HIV-1 viral load decreases and reaches the so called steady-state level ("set-point") that lasts for years (asymptomatic phase; Figure 1.2).

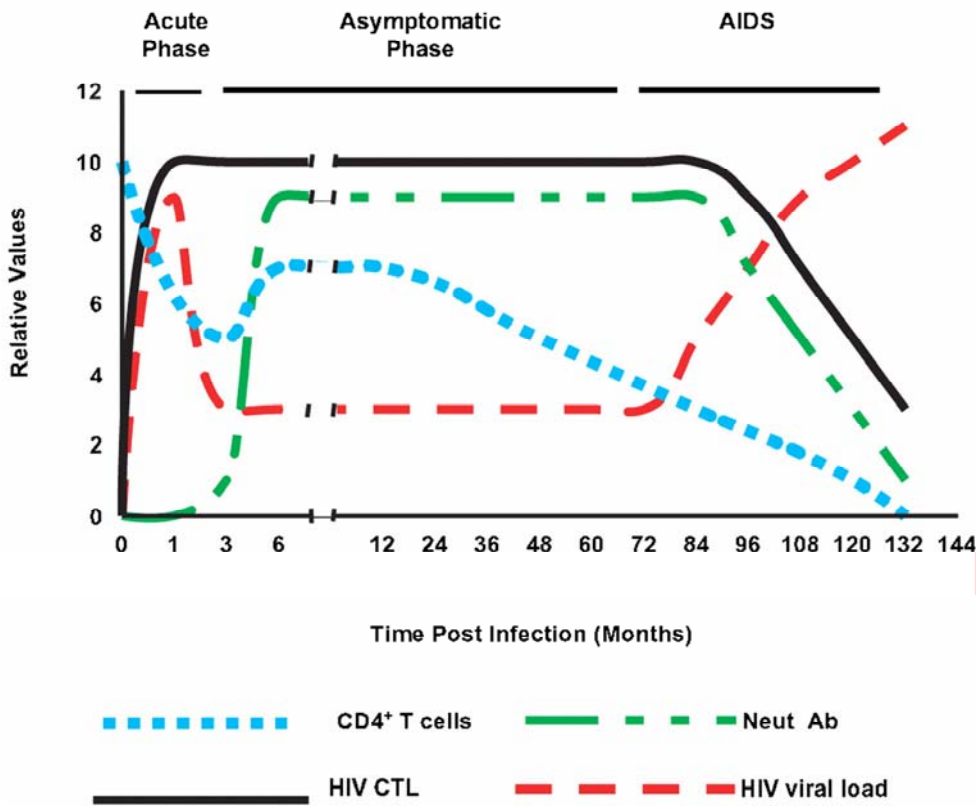


Figure 1.2: Kinetics of HIV disease (Adapted from Alimonty et al., 2003). Schematic representation of the typical course of HIV/AIDS disease progression, in the absence of anti-retroviral therapy. The initial spike in HIV load in the acute phase is accompanied by an increase in HIV-specific CTLs (cytotoxic T lymphocytes) and a decrease in the number of $CD4^+$ T cells. Within 1 to 2 months the viral load is reduced and maintained at a new lower threshold by the immune response. The lower viral load is concomitant with an increase in $CD4^+$ T cells, and at 2 to 3 months seroconversion occurs as HIV-specific antibodies appear. This new asymptomatic state is generally maintained for a number of years. Gradually the $CD4^+$ T cell counts decrease to below $200mm^3$ in the blood signaling the onset of AIDS and increased susceptibility to opportunistic infections. This is followed by an increase in the virus load, along with a decrease in the HIV-specific CTL, and neutralizing antibodies.

The "set-point" levels of viral load differ significantly between individuals. Identification of HIV-1 steady-state viral load prior to treatment initiation (base line measurement) represents a strong clinical marker of disease progression. A higher set-point is associated with faster disease progression to development of Acquired Immune Deficiency Syndrome (AIDS) and death, while lower set-points indicate slower disease progression to AIDS (Mellors et al., 1996; Wei et al., 1995). Serial measurements of HIV-1 viral load in combination with $CD4^+$ T cell counts are used routinely to monitor the disease phase of the patient, as well as the efficacy of antiviral therapy (Mellors et al., 1997; O'Brien et al., 1997).

There are a wide variety of commercially available assays for quantifying HIV-1 RNA. The majority of these assays are NAT that involve viral RNA isolation, subsequent target or signal amplification and detection of the amplification product. Three main methodologies have been FDA approved (Galli et al., 2005; Swanson et al., 2005) and are considered "gold standard' assays for HIV-1 viral load quantitation: the COBAS Amplicor HIV-1 Monitor™ 1.5 test (Roche Molecular Systems, Branchburg, N.J., USA), the NucliSens™ HIV-1 QT test (BioMérieux, Inc., Durham, NC) and the Versant HIV-1 RNA 3.0 test (Bayer Diagnostics, Tarrytown, NY). For over a decade these methodologies have undergone numerous modifications and evaluations aimed to improve their specificity for different HIV-1 subtypes (Antunes et al., 2003; Parekh et al., 1999; Respess et al., 1997; Swanson et al., 2005; Swanson et al., 2001). The current versions of the "gold standard" assays, listed above, and their new, recently developed modifications are reviewed below. The Amplicor ™ HIV-1 Monitor 1.5 assay (Roche Molecular Systems, Branchburg, N.J.) represents competitive quantitative reverse transcription polymerase chain reaction (CQ RT-PCR). The assay has two versions: a standard and ultrasensitive assay. The two

versions differ in their sensitivity and linear dynamic range. A standard version of the assay requires 0.35 ml of patient plasma for viral RNA extraction. Specimen preparation for an ultrasensitive version of the assay involves ultracentrifugation of 0.5 ml of plasma prior to extraction (Sun et al., 1998). HIV-1 RNA is reverse transcribed to produce single stranded complementary DNA (cDNA; RT step), which is subsequently amplified by PCR. The entire target amplification process (RT-PCR) is driven by rTth polymerase, which under certain buffer conditions combines the properties of both reverse transcriptase and DNA polymerase enzymes (Mulder et al., 1994; Respess et al., 1997). Primers and probes for the Roche Amplicor assay version 1.5 are specific for the p24 *gag* region of HIV-1. Quantitation of virus is based on the use of an internal quantitation standard (QS). The standard is an *in vitro* transcribed RNA molecule with a scrambled HIV-1 sequence. The QS RNA molecule has the same primer binding sites as HIV-1 RNA, but a different probe binding sequence to differentiate between the amplicons (Michael et al., 1999; Triques et al., 1999). A new, automated modification of the Roche Amplicor version 1.5 uses an automated Cobas AmpliPrep specimen preparation system and a COBAS Amplicor robotic Analyzer for RT-PCR and post-PCR detection (Berger et al., 2005; Berger et al., 2002). In brief, detection is performed by the probe-based capture of PCR product on magnetic particles and subsequent colorimetric detection of the captured molecules. The overall dynamic range of the COBAS Amplicor assay covers 5 logs of magnitude, with a lower limit of quantitation (LLQ) at 50 RNA copies/ml and the upper limit of quantitation (ULQ) at 750 000 RNA copies/ml (Erali and Hillyard, 1999; Swanson et al., 2005). In South Africa this assay is the “gold standard” test for HIV-1 viral load quantitation. The most recent development from Roche Molecular Systems is the COBAS TaqMan HIV-1 test. It makes use of real-time PCR technology and similarly to the Amplicor test version 1.5 involves viral RNA extraction, RT-PCR and real-time detection of amplification product.

Real-time RT-PCR and on-line detection are performed on the COBAS TaqMan 48 Analyzer using a fluorescently labeled TaqMan probe. The assay uses the highly conserved region of the HIV-1 *gag* gene in order to ensure equal detection specificity for various HIV-1 group M subtypes. Quantitation of viral copies is based on the use of an internal standard. The dynamic range of the COBAS TaqMan HIV-1 assay is 40 – 10,000,000 RNA copies/ml (Katsoulidou et al., 2006).

The NucliSens™ HIV-1 QT test (BioMérieux, Inc., Durham, NC) employs an isothermal target amplification system named nucleic acid sequence-based amplification (NASBA). Unlike PCR technology that requires temperature cycling, an isothermal amplification is performed at a single temperature, i.e., 41ºC for NASBA. NASBA is driven by simultaneous activity of three different enzymes: AMV (Avian Myeloblastosis Virus) reverse transcriptase, T7 RNA polymerase and RNase H. One of the two primers used for NASBA is usually modified by the addition of a T7 promoter sequence recognized by T7 RNA polymerase (Campton, 1991). Figure 1.3 illustrates the mechanism of amplification using NASBA. Quantitation of viral load with the NucliSens™ QT assay is based on the co-amplification of viral RNA with the three internal calibrators of a known concentration. Detection of the anti-sense RNA amplicons from both viral target and the calibrators utilizes ruthenium-labeled probes and a magnetic bead capture format. The signal from the probes is read and quantified by an electrochemiluminescence (ECL) reader (van Gemen et al., 1993; van Gemen et al., 1994). The primers used for the NucliSens™ QT assay are specific for a highly conserved region of the HIV-1 *gag* gene (Yen-Lieberman et al., 1996). HIV-1 RNA is extracted from 0.2ml of patient plasma, and the dynamic range of the NucliSens™ QT assay is from 80 to 5 000 000 RNA copies/ml (Swanson et al., 2005). Good performance characteristics of NucliSens™ QT test, in particular high sensitivity and

specificity have been demonstrated not only using plasma samples (Lujan-Zilbermann et al., 2003), but also on seminal plasma (Fiscus et al., 2000) and dried blood spots (Brambilla et al., 2003).

A new HIV-1 viral load assay - NucliSens™ EasyQ (version 1.1) has recently been developed by BioMérieux. The EasyQ test uses a single internal calibrator for quantitation of viral copies and a fluorescently labeled molecular beacon probe for detection of RNA amplicons (Figure 1.3). The NASBA reaction and real-time detection of amplicons is performed using the real-time EasyQ analyzer. This assay has a wider dynamic range than the NucliSens QT test (Weusten et al., 2002). Evaluation of the EasyQ test on HIV-1 subtype C samples in our laboratory showed good correlation with the COBAS Amplicor version 1.5 assay (Stevens et al., 2005b). The EasyQ assay has recently been implemented in South Africa for HIV-1 viral load monitoring in patients enrolled in the national ARV treatment program.

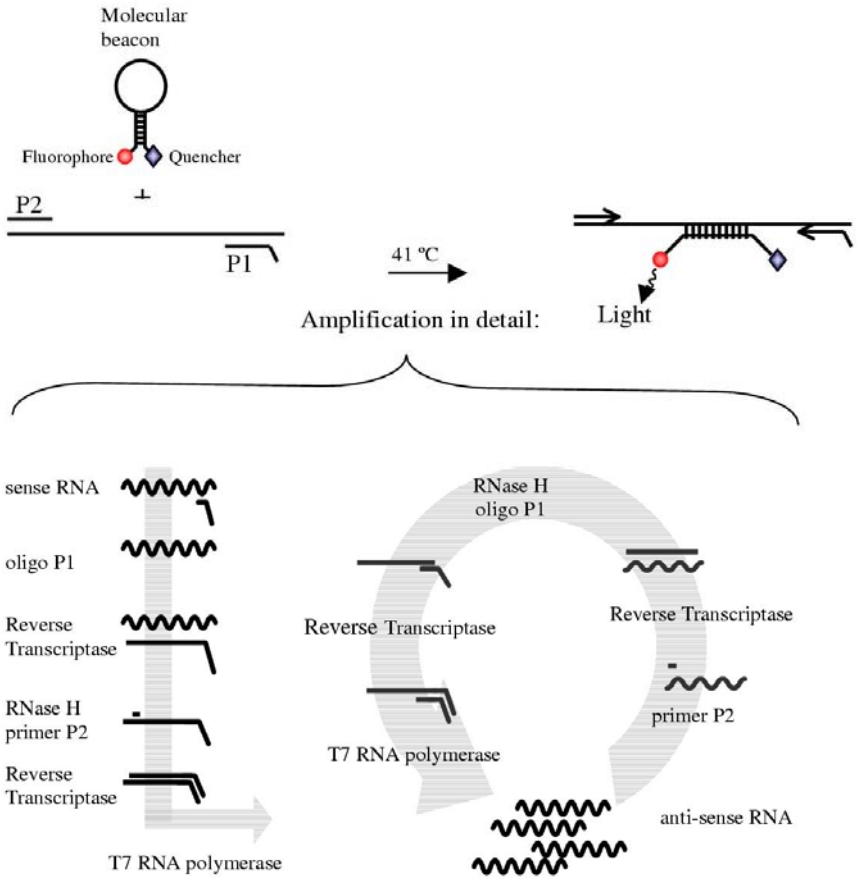


Figure 1.3: Schematic representation of the NASBA reaction and molecular beacon detection (diagram adapted from Polstra et al., 2002). The P1 (anti-sense) primer with the T7 promoter overhang binds viral RNA. AMV RT converts sense RNA into cDNA. The resulting viral RNA/cDNA hybrid is converted to the single stranded (ss) DNA by RNase H activity of the AMV enzyme that digests sense RNA. Anti-sense ssDNA is converted to the double stranded (ds) DNA by P2 (sense) primer and AMV. This dsDNA represents a template with a functional T7 promoter that can be used by T7 RNA polymerase to produce multiple copies of anti-sense RNA (linear phase of amplification). Each copy of anti-sense RNA binds P2 primer and serves as a target for the next round of amplification – the process described above gets repeated (cycling phase). The molecular beacon is a dual labeled fluorogenic probe for detection of the anti-sense RNA amplicons. In the presence of the target molecules the stem-loop structure of the probe opens and it hybridizes to the target. Upon hybridization, the fluorescent and quencher dye get separated and fluorescence levels significantly increase.

The Versant HIV-1 RNA 3.0 test (Bayer Diagnostics, Tarrytown, NY), also referred to as the Quantiplex HIV-1 RNA test is based on an isothermal (45ºC), branched DNA (bDNA) amplification of signal . At the initial step of the assay, virus particles are concentrated by centrifugation of plasma and the pellet lyzed by proteinase K and detergent to release RNA. Subsequent, linear signal enhancement by bDNA technology utilizes a combination of five types of oligonucleotide probes (Figure 1.4). Microtiter plates prepared for the assay have capture probes attached to the sides of the wells. Another set of capture probes – the capture extenders (CEs) have sequences complementary to the capture probes and to viral RNA. Isolated HIV-1 RNA is captured by hybridizing to CE probes. Next, the large number (>30) of viral RNA-specific probes – label extenders (LEs) also bind to the captured target. Each of the numerous LE probes are then hybridized to the longer probe called the pre-amplifier. The pre-amplifier probes in turn bind many amplifiers labeled with alkaline phosphatase (AP). The current version of the bDNA test also makes use of modified, novel nucleotides – isoG and isoC. These modified dNTPs are incorporated into the sequences of pre-amplifier, amplifier and AP-labeled probes. This minimizes background signal by reducing non-specific binding between different probes since isoC and isoG won't hybridize to the natural dCTP and dGTP. Detection is achieved by incubating the probe-target complex with the chemiluminescent substrate and measuring emitted light with a luminometer. The strength of luminescent signal is directly proportional to the amount of initial RNA target in the reaction. HIV-1 RNA is quantified using an external standard curve run in the same assay (Collings et al., 1997). The Versant HIV-1 RNA 3.0 test targets the highly conserved regions of the HIV-1 *pol* gene and requires 1ml of plasma for RNA isolation. The LLQ of the bDNA assay is set at <100 RNA copies/ml (according to exUSA and USA package inserts <50 RNA copies/ml and <75 RNA copies/ml, respectively) and the UQL of the assay is 500 000 RNA copes/ml

(Swanson et al., 2005). Several studies demonstrated highly significant correlation between the bDNA assay and the Ultra sensitive Roche Amplicor version 1.5 assays. The Versant version 3.0 assay also showed good sensitivity, dynamic range and excellent reproducibility (Berndt et al., 2000; Elbeik et al., 2000; Erice et al., 2000; Galli et al., 2005).

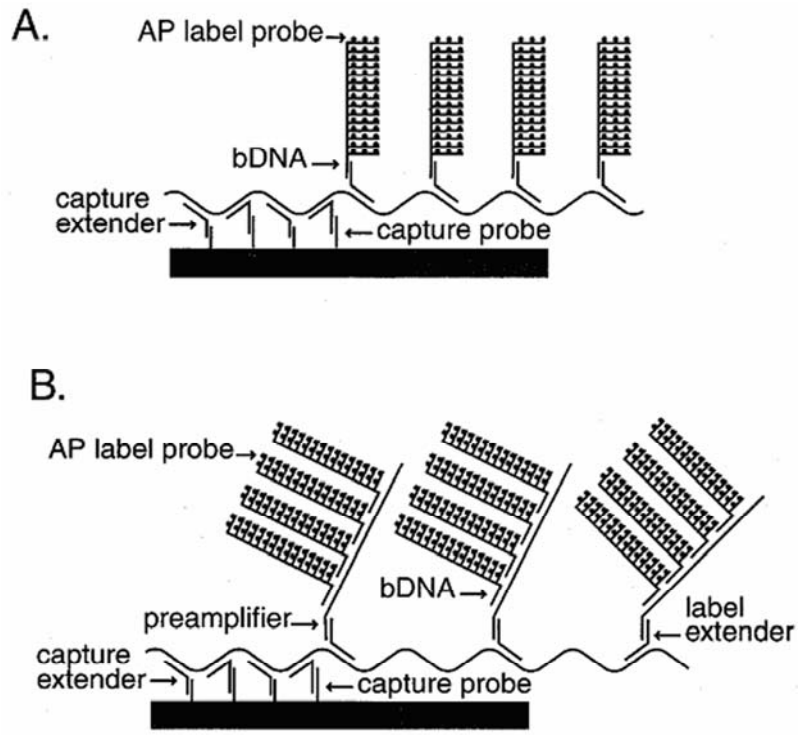


Figure 1.4: Basic bDNA assay components (Figure and legend adapted directly from Collings et al., 1997). (A) First generation assay; (B) second and third generation assays. The preamplifier (heavy lines) is unique to the second and third generation assays.

Overall, all three "gold" standard assays are highly correlated and demonstrate excellent performance characteristics: good reproducibility, high sensitivity (50-100 RNA copies/ml), 100% specificity and are capable of quantifying group M subtypes (A-G) of HIV-1.

There are also other NAT based commercial assays available for monitoring HIV-1 viral load that are in wide spread use but do not have FDA approval. The LCx® HIV-1 RNA quantitative assay (Abbott Laboratories, North Chicago, IL) utilizes competitive RT-PCR followed by microparticle enzyme immunoassay detection on the LCx Analyzer. HIV-1 RNA is purified from 1 ml of plasma using the Qiagen sample preparation kit (QIAGEN® GmbH, Germany). The assay makes use of an internal control and an externally run calibration curve for quantitation of HIV-1 RNA copies. The dynamic range of the assay is 50 - 1000000 RNA copies/ml. Primers and probes are specific to a highly conserved *pol* IN region of the HIV-1 genome (Johanson et al., 2001). The LCx® HIV-1 RNA quantitative assay shows good specificity, good correlation of quantitation results with the "gold" standard tests for all subtypes of group M and more efficient quantitation of group O samples than the "gold" standard tests (Berger et al., 2001; Katsoulidou et al., 2004; Swanson et al., 2005; Swanson et al., 2001).

A recent development from Abbott (Abbott Molecular Inc., Des Plaines, IL) is the automated, high throughput (96 samples) RealTi*me*™ HIV-1 assay. For this assay viral RNA is extracted from 1ml of plasma using the Abbott *m*2000*sp*™ automated sample preparation system and quantitative real-time RT-PCR is performed on the *m*2000*rt*™ instrument (Abbott Molecular Inc.). The RealTi*me*™ HIV-1 assay provides wide linear dynamic range from 40 to 10 000 000 RNA copies/ml in the presence of an internal control. Primers and probes used in the assay are specific to the *pol* IN region of HIV-1. Evaluation of the RealTi*me*™ HIV-1 assay using a panel of 91 plasma specimens from Brazil demonstrated equivalent quantitative performance with LCx HIV assay on subtypes B, C, F and recombinant B/F strains. Good agreement between the new assay and the LCx

HIV assay was demonstrated for this panel using the Bland-Altman model (Swanson et al., 2006).

The Gen-Probe HIV-1 viral load assay (Gen-Probe Inc., San Diego, CA) uses transcription-mediated amplification (TMA) and detection by chemiluminescence. TMA technology is almost identical to NASBA (Figure 1.3) with a single difference: TMA makes use of the AMV enzyme which possesses RNase H activity. The dynamic range of the assay is from 25 to 100 000 RNA copies/ml. The Gen-Probe viral load targets the conserved region of the *pol* gene of HIV-1 which allows good detection and quantitation of all subtypes from group M of HIV-1 and even rarer group O strains. The assay has shown to be a good alternative for monitoring HIV-1 viral load in plasma and other types of biological specimens, in particular breast milk and genital swabs (De Vange Panteleeff et al., 2002; Emery et al., 2000; Neilson et al., 1999).

The Retina HIV-1 viral load assay (Primagen Holding B.V., Amsterdam, The Netherlands) uses NASBA technology for target amplification and molecular beacon probes for real-time detection of amplicons. Reproducible sensitivity of the assay is 500 RNA copies/ml. Primers and probe are specific for a highly conserved region of the HIV-1 genome, the long terminal repeat (LTR) region. The assay can reliably detect and quantitate viral RNA in group M, N and O subtypes of HIV-1 (de Baar et al., 2000; de Baar et al., 2001).

As described above, NAT based HIV-1 RNA quantitation tests reveal high performance characteristics and are most useful for monitoring patient viral load in response to ARV therapy. They provide reasonably high throughput and are semi- or fully automated. However, these “high tech” assays remain largely unaffordable for the developing world. Implementation of NAT requires a laboratory set-up with preferably unidirectional work

flow with dedicated areas for sample preparation, amplification and detection. Moreover, a set of specialized equipment and costly reagents make these assays quite expensive for the resource constrained settings. An overview of the main technical characteristics of FDA approved and some recently developed NAT based viral load assay are summarized in Appendix A.

In an attempt to provide more affordable, simpler options for developing world scenarios certain commercial companies have designed non-NAT based approaches to develop less complex, affordable alternatives to HIV-1 viral load monitoring. The Perkin-Elmer heat-denatured (HD), signal-boosted HiSens HIV-1 p24 assay and the Cavidi ExaVir® Load Quantitative HIV-RT assay are examples of such approaches that do not use viral RNA as a target. Indirect measurement of HIV-1 viral load is performed by quantitation of p24 antigen in the Perkin-Elmer HiSens p24 assay and by estimation of HIV-1 reverse transcriptase (RT) activity in the Cavidi RT assay (Award, et al., 1997; Garcia, et al., 1998; Ledergerber, et al., 2000; Schüpbach, et al., 1996). Both assays utilize less expensive enzyme-linked immunosorbent assay (ELISA) detection formats and can be performed in the less sophisticated laboratory set-up. Evaluation of these more affordable alternative HIV-1 viral load tests in a South African setting represents one of the primary objectives of this study. A more detailed description of methodologies used for these non-NAT based alternative viral loads are reviewed in chapter 3.

1.3 Identification of Acute HIV-1 infection (AHI)

The typical course of HIV-1 infection is characterized by an acute/primary phase that usually lasts for a few weeks, followed by an asymptomatic phase that usually lasts 3- 10 years, which eventually leads to immune collapse termed the Acquired Immune Deficiency

Syndrome (AIDS) (Figure 1.2). The acute phase of HIV infection is also described as the HIV antibody-negative stage of the disease. During the acute phase the absence of host immune responses favors uncontrolled viral replication. Consequently, very high levels of viremia occur, and there is increased shedding of virus from mucosal sites. A reduction in viremia approximately 30 days post infection occurs when immune responses to the virus emerge and anti-HIV antibodies become detectable (seroconversion). A gradual reduction in viremia follows and the establishment of a so called "set-point" (Figure 1.2) indicates the start of a clinically asymptomatic phase (Alimonty, et al., 2003; Pilcher, et al., 2004). Between 1-4 weeks post-infection some individuals in the acute phase develop influenza-like symptoms that are characteristic of most febrile illnesses (Coco, 2005; Schacker et al., 1996). This combination of non-specific symptoms observed during the acute phase of HIV-1 infection has been termed the acute retroviral syndrome (Kahn and Walker, 1998).Thus, AHI remains largely undiagnosed (>90%) due to the absence of specific clinical manifestations (Pilcher, et al., 2004; Coco, 2005).

From a public health point of view early diagnosis of AHI in a high prevalence population could potentially decrease transmission of the infection and the epidemic spread of the disease. Several studies of acute HIV-1 show that viral load in semen (Vernazza et al., 1994; Vernazza et al., 1997) and female genital fluids (Hart, 1999) can be higher than the extremely high viral load detected in plasma. High viremia is also associated with strikingly elevated genital and rectal shedding of virus during acute HIV-1 infection (Dyer et al., 1997; Pilcher et al., 2004b). The characteristic high plasma viral load and elevated shedding from mucosal and genital areas identify the period of "hyper infectiousness", which accounts for up to a 20-fold increase in sexual transmission rates (Pilcher et al., 2004b). Hyper infectiousness in the acute phase in combination with unawareness of ones

HIV-1 status has largely contributed to the rapid global spread of HIV-1 infection (Coco, 2005; Leynaert et al., 1998). Diagnosis of acute HIV-1 infection is also critical for prevention of mother-to-child transmission (MTCT) relying on early administration of treatment both in neonates and mothers. In addition, identification of HIV-1 during the first few weeks of infection can substantially increase the safety of donated blood and blood products from acutely infected HIV infected individuals, and helps to prevent this route of viral transmission (Fiebig et al., 2003). Some studies demonstrated that initiation of treatment during the acute phase of HIV-1 infection may dramatically improve the outcome of ARV therapy by directly targeting latent reservoirs of virus (Lafeuillade et al., 2003) and facilitating maximum anti-HIV immune responses that also help to clear viral reservoirs (Oxenius et al., 2000; Rosenberg et al., 2000).

In general, the diagnosis of HIV infection is based on the identification of viral markers that appear in an individual's blood or other body fluids in the following chronological order: viral RNA, DNA, p24 antigen, and antibody to HIV antigens. In the context of HIV detection, the time interval before antibodies appear is known as the serological "window period". This period is characterized by seronegativity, high levels of viremia detectable by the presence of viral RNA and p24 antigen, and variable $CD4^+$ T lymphocyte levels. In the majority of individuals, antibodies to HIV are usually detected within 1-2 months post infection regardless of the method used (Constantine et al., 1992). Commercial HIV-1 diagnostic tests can be classified depending on the type of viral marker used as a detection target to identify the infection. Additionally, irrespective of the viral markers used HIV diagnostic assays fall into two major categories: (i) screening (diagnostic) assays, which are designed to identify all infected individuals; and (ii) confirmatory (supplemental) assays, which are designed to differentiate between false positive and truly positive results

obtained using the screening assays. Correspondingly, screening assays are characterized by a high degree of sensitivity (low false negative rate) and confirmatory assays are designed to provide high specificity (low false positive rate). In most cases both categories of assays are used consecutively to ensure highly accurate and reliable diagnosis of HIV infection. Often, for cost saving purposes a combination of different screening assays is used in a specially designed confirmatory algorithm (e.g. rapid assays are usually confirmed using either third or fourth generation enzyme-linked immunosorbent assays (ELISA) assays) (Constantine and Zink, 2005).

Rapid HIV-1 serologic tests have been designed to detect anti-HIV antibodies in oral mucosal transudate, whole blood, serum and plasma. These tests are most useful for diagnosing HIV infection after seroconversion. These assays are suitable for POC testing as they are quick (~20- 45 minutes), generally easy to perform (in 1-2 steps) and do not require a laboratory setting, specialized equipment or skilled personnel. Rapid assays employ different technologies: (i) flow-through; (ii) lateral flow; or (iii) agglutination assays (Aaron et al., 2006; Gupta and Chaudhary, 2003; Sudha et al., 2005). A wide variety of rapid tests are commercially available (Respess et al., 2001). OraQuick and OraQuick ADVANCE rapid HIV-1/2 antibody tests (OraSure Technologies, Inc., Bethlehem, PA, USA) for oral transudate received the US Food and Drug Administration (FDA) approval based on their performance characteristics (Aaron et al., 2006; Cohen et al., 2003; Liang et al., 2005; Reynolds and Muwonga, 2004). According to the WHO recommendations (WHO/UNAIDS, 1999) rationalized testing strategies (algorithms) based on employing a combination of 2-3 consecutive rapid tests (rapid screening test followed by other rapid, confirmatory tests) are often used. These confirmatory testing algorithms reduce the number of indeterminate and incorrect results, but increase the cost of testing

(Menard et al., 2005; Respess et al., 2001; Wright et al., 2004). The use of rapid tests is standard of care in resource limited settings where laboratory infrastructure, stable supply of electricity and trained personnel are omnipresent. In South Africa the most widely used HIV rapid tests are the Determine HIV-1/2 (Abbott Laboratories, USA), Uni-Gold HIV (Trinity Biotech plc, Ireland) and Capillus HIV-1/2 (Cambridge Diagnostics, Galloway, Ireland). Determine HIV-1/2 and Uni-Gold HIV represent lateral flow based anti-HIV antibody rapid tests, and the Capillus is a particle agglutination test. These rapid tests show high sensitivity and specificity and are often used in alternative screening as well as in confirmatory HIV testing algorithms to provide more accurate HIV diagnostics (De Baets et al., 2005; Ferreira Junior et al., 2005; McKenna et al., 1997; Menard et al., 2005; Phillips et al., 2000; Ramalingam et al., 2002; Soroka et al., 2003).

ELISAs or enzyme immunosorbent assays (EIA, term is used interchangeably) for detection of anti-HIV IgG and IgM antibodies are often referred to as third generation assays. Detection of IgM which appears prior to IgG allows the early diagnosis of HIV. Antibody (Ab) ELISA assays can be performed on oral fluid, urine and blood samples. ELISA configuration of the third generation assays makes use of so called antigen sandwich detection where the bivalent anti-HIV antibody binds to both the capture antigen attached to the solid support and the detector antigen labeled with an enzyme. Generally, third generation assays have slightly higher sensitivity and specificity of antibody detection than rapid tests. During seroconversion, the third generation assays can detect anti-HIV antibody 2-8 days earlier than the rapid tests (Makuwa et al., 2002). These assays provide cost-effective and high throughput diagnostics for HIV. The third generation Vironostika Uni-Form II plus O assay (BioMérieux, The Netherlands) is a widely used assay in South Africa for HIV-1diagnosis. This anti-HIV Ab ELISA assay is highly sensitive and shows

good specificity across different HIV-1 groups M and O subtypes (Aboud et al., 2006; Murphy et al., 2003; van Binsbergen et al., 1996; van Binsbergen et al., 1997; van Binsbergen et al., 1999). The Vironostika Ab ELISA assay in combination with the Orasure HIV-1 oral specimen collection device was used to estimate the HIV-1 prevalence in the South African population (Connolly et al., 2004).

Another type of laboratory based diagnostic assay allows combined detection of HIV antigen (Ag) and antibody (Ab). These assays are also known as fourth generation assays (Weber et al., 1998). There are a variety of commercially available fourth generation assays including AxSym HIV Ag/Ab Combo (Abbott Laboratories, IL, USA), Enzygnost HIV Integral (Dabe Behring, Penzberg, Germany), Genscreen Plus HIV Ag/Ab (Bio-Rad, Marnes La Coquette, France) and Murex HIV Ag/Ab Combo (Abbott, Dartford Kent, England), amongst others. Due to the detection of HIV p24 antigen in seroconversion samples, combined Ab/Ag tests reduce the detection window by approximately 6 days, to approximately 14-15 days post infection, when compared to the third generation assays (Aghokeng et al., 2004; Ly et al., 2004). In South Africa one of the most widely used fourth generation ELISA assays is the Murex HIV antigen/antibody assay (Abbott Murex, UK). The assay was shown to be one of the most sensitive among the Ab/Ag combined assays allowing detection of early seroconversion samples (Aboud et al., 2006; Ly et al., 2004; Ly et al., 2001).

Verification of the results obtained with Ab and Ab/Ag ELISA assays is performed using the most common serologic confirmatory assays like HIV Western blots (WB) and Line Immuno Assays (LIA). Generally, in the developed countries the Western blot is the most widely accepted confirmatory assay and may be considered as the “gold standard” assay for detection of anti-HIV antibodies and validation of HIV results (Constantine and Zink,

2005). Western blots detect reactivity of the human antibodies against at least 10 different viral antigens. Criteria for interpretation of Western blot results may differ in different laboratories and in different kit formats.Generaly, reactivity to at least two of the following antigens: gp160/120, gp41, or p24, is required to identify a reaction as positive. Criteria for interpretation of WB results suggested by the Centre for Disease Control (CDC, USA) differ from those recommended by WHO (US Department of Health and Human Services, Centers for Disease Control. Interpretation and use of the Western blot assay for serodiagnosis of human immunodeficiency virus type 1 infection; WHO, proposed criteria for interpreting results from Western blot assays for HIV-1, HIV-2 and HTLV-I/HTLV-II.). The absence of any reactivity in WB indicates negative HIV results. Although, WB and LIA confirmatory assays are commercially available in a kit form they are costly, and require expensive and sophisticated equipment (Cohen et al., 2003; Ferreira Junior et al., 2005; Makuwa et al., 2002), which also makes them unaffordable for developing countries. WHO and CDC recommendations for HIV testing strategies, HIV screening and confirmatory algorithms differ for the developing and developed countries (http://www.cdc.gov/mmwr/pdf/rr/rr/1519.pdf).

The rapid and third generation EIA assays detect anti-HIV antibody, which makes them inappropriate for the diagnosis of the acute, antibody-negative phase of HIV infection (Figure 1.5). Even though the fourth generation assays detect p24 antigen, their sensitivity of detection is significantly lower when compared to p24 Ag ELISA assays (Ly et al., 2004). Therefore, diagnosis of acute HIV-1 infection relies on detection of p24 antigen (p24 Ag EIA) and/or HIV-1 RNA or proviral DNA. Both p24 antigen and HIV RNA increase in parallel during viral replication. However, due to higher sensitivity of RNA amplification tests this marker is detected earlier than p24 antigen. A number of

commercial p24 Ag EIA assays are available including the FDA approved Abbott HIV-1 Ag monoclonal and Coulter HIV-1 p24 antigen assay. On average, limits of sensitivity of p24 Ag ELISA assays are 3- 10pg of p24 antigen per ml (Fransen et al., 2001; Weber et al., 1999). Viral RNA can be detected during the first 2 weeks after infection, before p24 antigen is detectable (Figure 1.5).

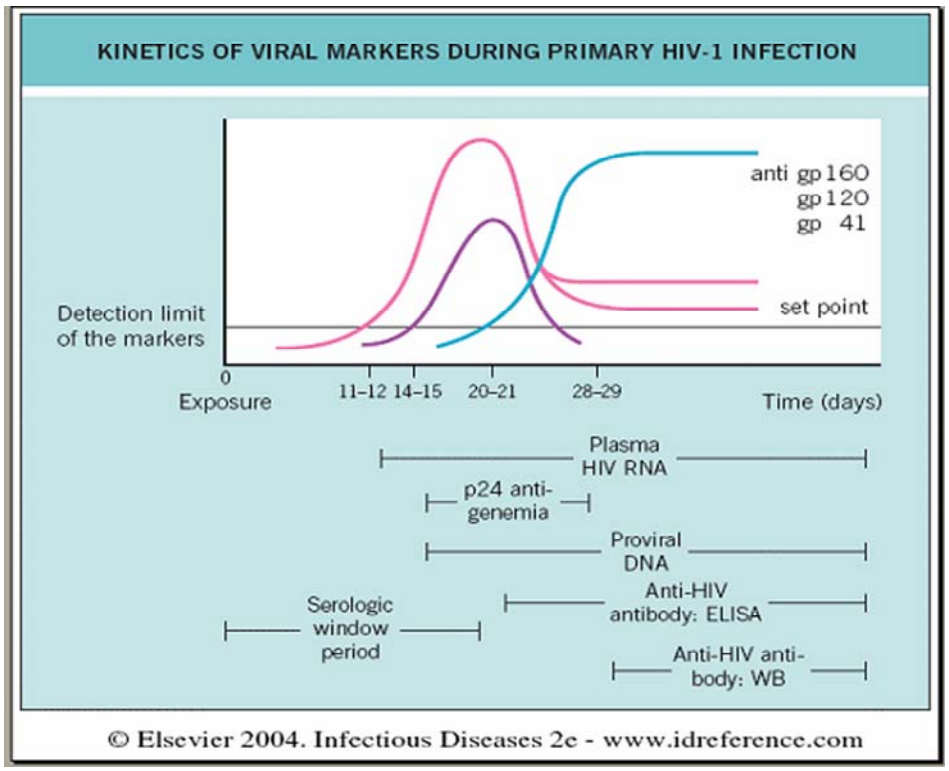


Figure 1.5: Kinetics of viral markers during primary HIV-1 infection (adapted from Cohen and Powderly, 2003). Keys: Pink = plasma HIV RNA; Purple = p24; Blue = anti-HIV antibody. Serologic window period is ~ 3 weeks post-infection; after this period anti-HIV Ab(s) become detectable. Detection of HIV-1 RNA can reduce the detection window to day 11-12 post-infection and provide means for earliest diagnosis of AHI.

NAT for determination of viral RNA are mainly used for monitoring HIV-1 viral load. Specimen pooling strategies combined with reverse transcription polymerase chain

reaction (RT-PCR) have been used to reduce costs of screening of acute HIV-1 infection in low and high prevalence populations (Pilcher et al., 2002; Stevens et al., submitted). Approved commercial assays for HIV-1 RNA quantitation were discussed previously (section 1.2). Studies conducted by different groups come to the mutual conclusion that p24 Ag ELISA assays are less sensitive, but more specific for detection of acute HIV-1 infection when compared to viral RNA tests (Daar et al., 2000; Hecht et al., 2002). Reduced specificity of NAT is due to false positive results. Ideally, NAT for HIV-1 RNA could provide the earliest diagnosis of the acute phase by further reducing the detection window when compared to the p24 Ag ELISA (Figure 1.5).

However, irrespective of the high (100%) sensitivity (Daar et al., 2000; Hecht et al., 2002) of current HIV-1 RNA assays, specialized laboratory set-up, expensive equipment and cost of testing makes them less appropriate for routine screening of acute/primary HIV-1 infection in resource constrained settings. The most appropriate assay for diagnosing incidence of AHI in South Africa would be a POC assay based on nucleic acid amplification technique(s) (NAAT). To our knowledge, such an assay that will allow detection of HIV-1 RNA in a POC testing format is not commercially available at present.

CHAPTER 2

DEVELOPMENT AND EVALUATION OF AN HIV-1 QUANTITATIVE REAL-TIME RT-PCR ASSAY USING THE LIGHTCYCLER® PLATFORM

This chapter is dedicated to the development and preliminary evaluation of a new, in-house quantitative real-time RT-PCR assay for monitoring HIV-1 viral load in plasma. The initial development of the assay, including basic optimisation of RT-PCR conditions and other technical parameters, was performed using the LightCycler® platform version 1.2 (Roche Applied Science, Mannheim, Germany) and the FRET probes for detection of amplicons. Recombinant plasmids with a cloned external standard (ES) for quantitation and an internal control (IC) were produced for the assay. Evaluation of assay performance was done using an RNA transcript of the ES and the BBI panel for HIV-1 subtype C infected plasma. The developed viral load assay was subsequently transferred to the new, more advanced version of the LightCycler® instrument (version 2) as this became available in South Africa. Following the technology platform transfer two main modifications were applied to the original "FRET assay": (i) RT-PCR was performed in a larger reaction volume (50 µl instead of 20 µl) and (ii) the FRET probe detection format was replaced with the LUX™ (Light upon Extension) primer. For simplicity the initial version of the developed viral load assay that uses FRET hybridization probes and the final version of the assay that uses a LUX™ primer detection format is referred to as the FRET assay and the LUX assay, respectively. Initial evaluation of the LUX assay was performed using ES RNA transcript

and VQA copy controls. Preliminary clinical evaluation was performed using plasma samples obtained from known HIV-1 seronegative patients (n = 50), and clinical specimens from a random group of HIV-1 positive patients (n = 142) and a cohort of 55 HIV-1 positive patients on HAART followed-up longitudinally (n = 266; 458 plasma specimens in total).

2.1 Introduction

A comprehensive review of all aspects and variations of real-time polymerase chain reaction (PCR) technology is beyond the scope of this thesis and therefore this introductory section will focus only on the aspects of this technology that are related to the current investigation. Real-time PCR is the greatest innovation that has been recently applied to PCR technology. Analyzing PCR product on-line, during the amplification process is termed "real-time" PCR. Real-time PCR is made feasible by a new approach to amplicon detection and modifications made to the conventional thermal cycler (Wittwer et al., 1997a). Non-sequence-specific detection of amplicons in real-time PCR is performed using fluorescent DNA dyes. The most widely used fluorophore is SYBR Green I dye that intercalates into double stranded DNA molecules and emits much higher levels of fluorescence than the unbound dye (Morrison et al., 1998; Pfaffl and Hageleit, 2001). Sequence-specific detection of PCR product is achieved by using oligonucleotide probes or modified primers that carry a fluorescent label. Although, numerous probes and primers differ in their design, there are two ways of generating a fluorescent signal: the separation of a fluorophore from a quencher or the association of a donor and acceptor dye to generate a fluorescent signal by energy transfer. Among probes labeled with a fluorescent and a quencher dye are TaqMan™ probes (Desire et al., 2001; Holland et al., 1991; Klein et al., 2003) and TaqMan™ probes with a 3' minor groove binding (MGB) group added (de Kok et al., 2002), Scorpion™ primers (Saha et al., 2001), Light upon Extension (LUX™)

primers (Nazarenko et al., 2002b; Nazarenko et al., 2002a), MGB Eclipse™ (Afonina et al., 2002) and double stranded "Yin-Yang" probes (Li et al., 2002). Fluorescent resonance energy transfer (FRET) hybridization probes (Figure 2.1) are labeled with a donor and an acceptor dye and represent the detection format most commonly used on the LightCycler® platform (Farcas et al., 2006; Lay and Wittwer, 1997; Makinen et al., 2002). LUX™ primers represent a recent advance in real-time detection technology. This detection format includes one single-labelled, self-quenched primer and an unlabelled counterpart. Figure 2.2 demonstrates the mechanism by which structural changes of the LUX primer during the steps of each PCR cycle allows for controlling the intensity of fluorescence. Although DNA-binding dyes are less expensive, the use of fluorescently labelled probes allows better specificity of detection and multiplex, simultaneous detection of multiple targets (Elenitova-Johnson et al., 2001).

Real-time PCR instruments combine features of a conventional thermocycler and a fluorimeter. Currently, there are a wide variety of real-time PCR instruments on the market (Bustin, 2002; Gibson, 2006). Among the first commercially available technologies were the ABI Prism instruments from Applied Biosystems, iCycler from BioRad and the Roche LightCycler® system. The LightCycler® (Roche Applied Science, Mannheim, Germany) is one of the fastest real-time PCR instruments. PCR reactions using the LightCycler® system are performed in glass capillaries placed in a rotating carousel (Wittwer et al., 1997b). The system uses pumped air for rapid heating and cooling of the reaction chamber that holds the carousel with 32 capillaries. A PCR cycle can be completed in 15-20 seconds. The LightCycler® system has blue LED light source for fluorescence excitation.

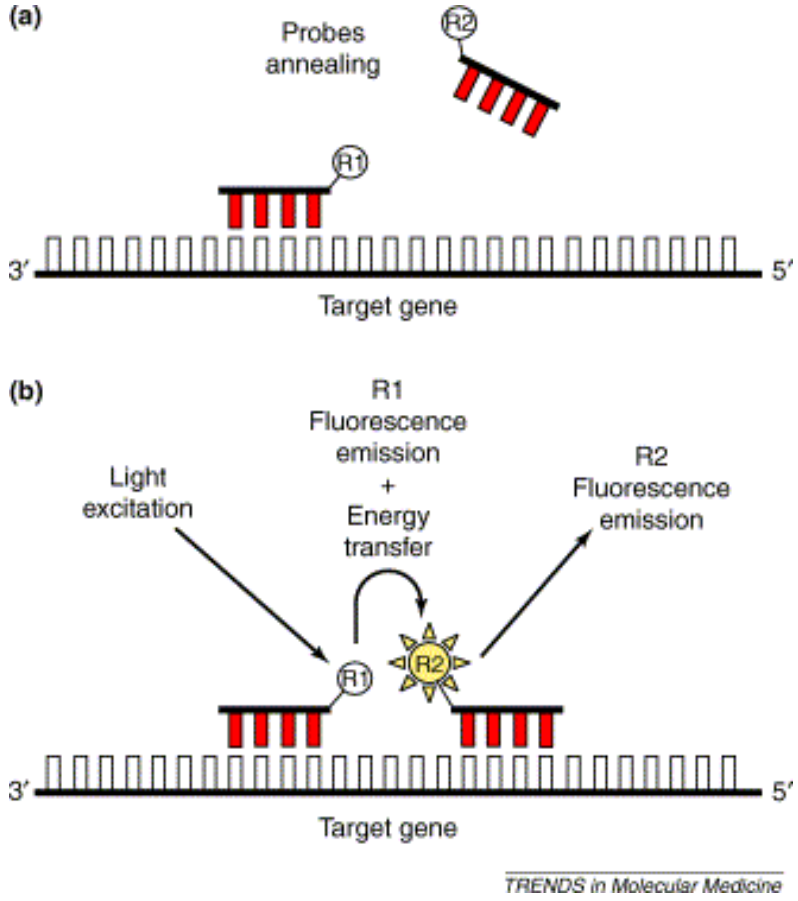


Figure 2.1: FRET Probes (Diagram adapted from Mocellin et al., 2003). (a) FRET detection format uses a pair of oligonucleotide probes: one probe labeled at the 3' end with a donor dye (R1) and another probe carrying an acceptor dye at the 5' end (R2). (b). during the annealing step of the PCR cycle both probes hybridize to the target sequence in a head-to-tail orientation bringing the donor and the acceptor dyes in close proximity of each other. The donor dye (usually FAM or Fluorescein) is excited by the light source (LED) of the real-time instrument, the energy from the excited donor fluorophore gets transferred to the acceptor dye and it in turn emits fluorescence recorded as a signal by the instrument.

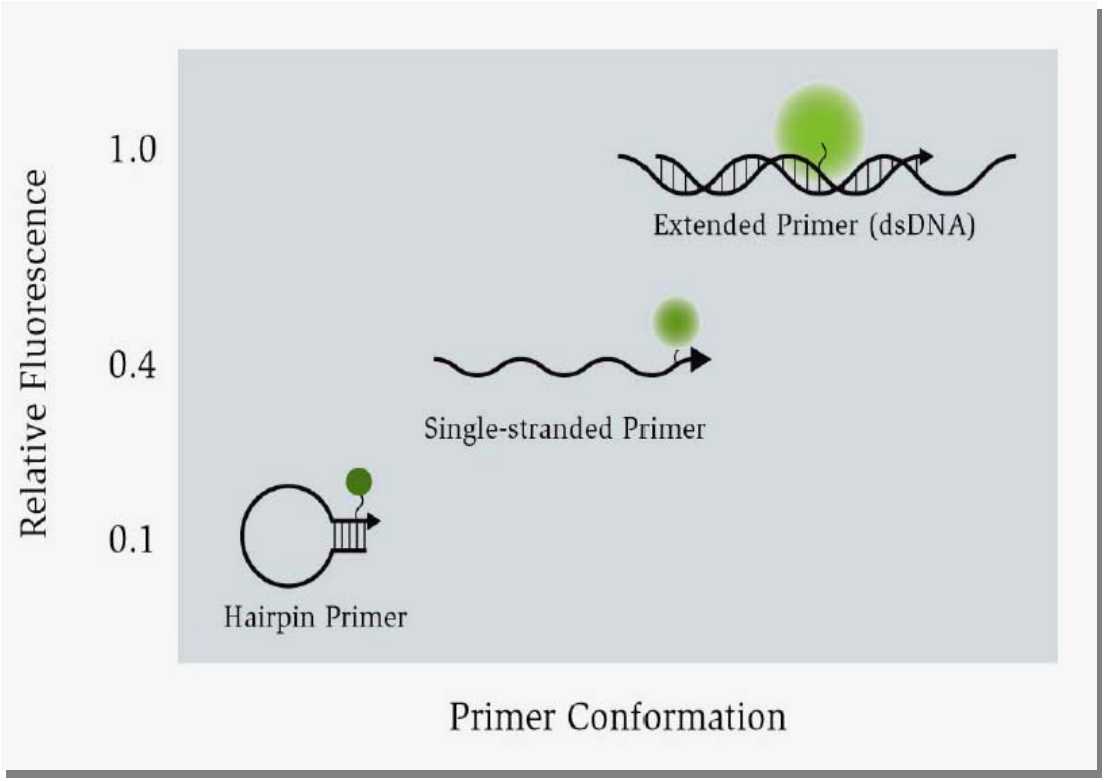


Figure 2.2: Schematic representation of the LUX™ Primer (Diagram adapted from: http://www.wzw.tum.de/gene-quantification/qpcr2004/pub/Nickson.pdf). Changes in emission of fluorescence are affected by the primary and secondary structure of a LUX™ primer. The labelled 3'-end of a LUX primer requires guanosine bases near the conjugated fluorescent tag. The 5'-end of this fluorogenic primer is modified by the addition of a short sequence that is complementary to the labelled 3'-end of the primer. This 5' tail allows a LUX™ primer to assume a hairpin configuration, which causes a fluorescence quenching effect. During PCR, when a fluorogenic LUX™ primer binds to the complementary sequence and becomes linear, the fluorophore is de-quenched and the levels of fluorescence increase by 10-fold.

Earlier versions of the LightCycler® instrument had three detection channels and could only accommodate small volume (20 µl) capillaries. The latest LightCycler® instrument, version 2.0, allows the use of 20 µl and 100 µl capillaries and has six detection channels: 530 nm (FAM, Fluorescein, SYBR Green), 560 nm (HEX, VIC), 610 nm (LightCycler (LC) Red 610), 640 nm (LC Red 640) and 710 nm (LC Red710). Similarly to other real-

time PCR instruments, the LightCycler® is operated through a computer system and specially designed software. Data analysis (qualitative or quantitative) is performed after completion of PCR using the LightCycler® software (Roche Applied Science, Mannheim, Germany) (Gibson, 2006).

Real-time detection systems have simplified and improved quantitative PCR. Conventional competitive quantitative PCR employs time consuming gel electrophoresis followed by less accurate densitometry analysis or probe based color detection for an end-point quantitation of product (Schutten et al., 2000). Real-time PCR platforms monitor fluorescent signals during each PCR cycle and the integrated software identifies the threshold cycle (C_T) within the exponential increase phase of PCR. The (C_T) value is the cycle at which the fluorescence acquired during the log-linear phase of amplification is significantly higher than the baseline (background) level. Theoretically, the threshold cycle is inversely correlated to the initial (input) amount of template molecules and represents a unique signature of template concentration. There are two "methods" of quantitation using real-time PCR – relative and absolute and both methods are based on identification of the C_T value. Relative quantitation is mostly used to study gene dosage or gene expression levels in comparison to the various housekeeping genes. The quantitation values are normally expressed as a ratio of the C_T of a housekeeping gene and the target gene. Absolute quantitation, or recently referred to as the standard curve quantitation method, is based on the use of an external quantitation standard. An external standard (ES) is defined as a sample of known concentration. Serially diluted ES samples (at least three different concentrations) are included in each run to determine the concentration of unknown samples. The integrated software of a real-time PCR system constructs a standard curve plotting the known concentration values of ES on the X-axis and the extrapolated,

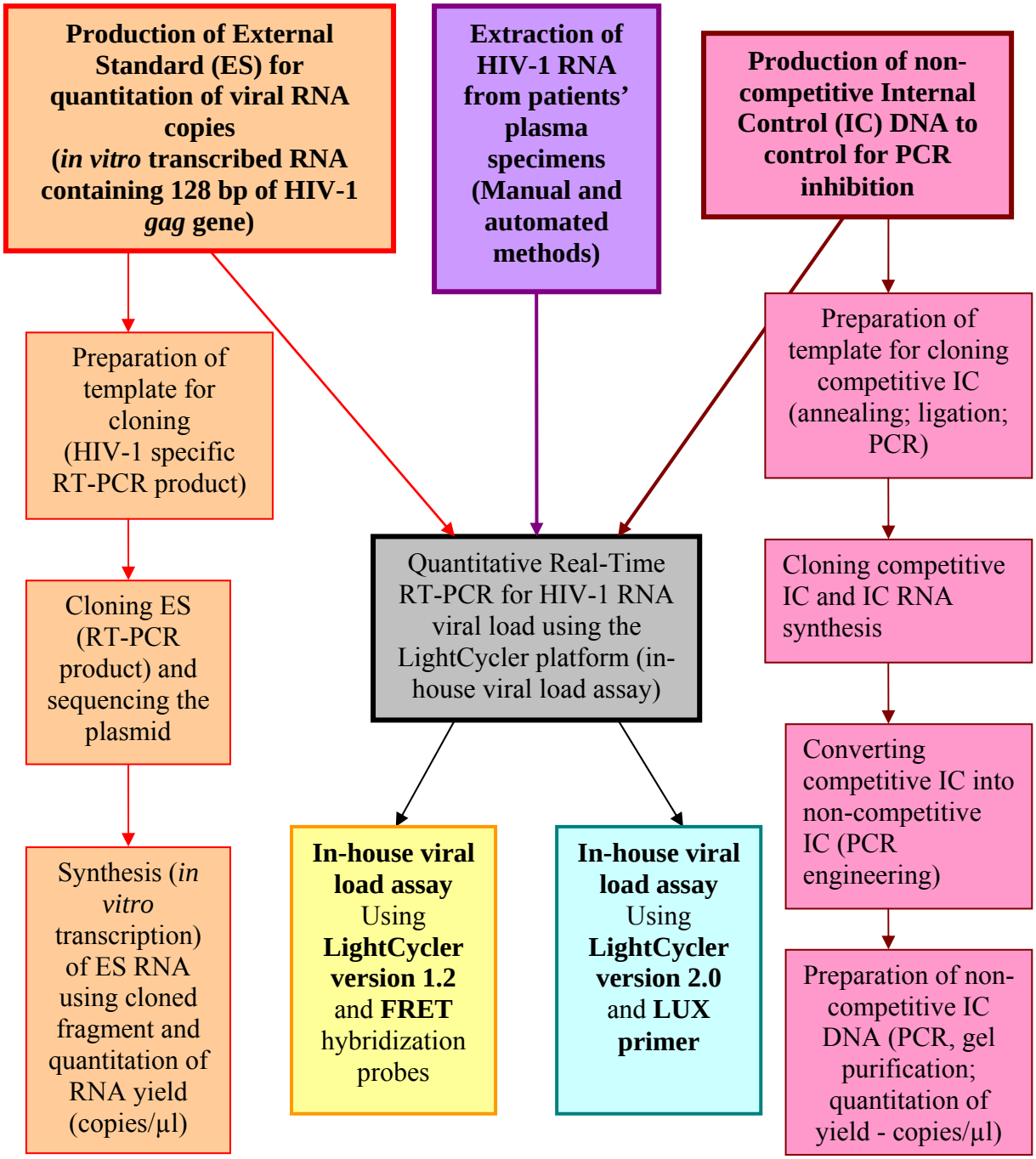
corresponding C_T values on the Y-axis. Therefore, the resulting standard curve reflects the linear relation between the template input and the C_T value. The concentration of template in an unknown sample is deduced by plugging its C_T value into the standard curve (Bustin and Nolan, 2004; Higuchi et al., 1993; Schmittgen et al., 2000; Wittwer). The concentrations obtained using an absolute quantitation method are usually expressed in weigh units or copy numbers of molecules per unit of volume, e.g. 400 pg/ml or 50 RNA copies/µl. In comparison to the end-point quantitative analysis of a conventional PCR, real-time quantitation is performed in a log-linear phase of exponential product accumulation and hence it is more accurate, sensitive and has a wide dynamic range.

Nucleic acid extraction (manual or automated) represents an important and certainly the most time consuming step of real-time PCR based assays. Several automated and manual extraction technologies are available in South Africa. The QIAamp® Viral RNA mini kit (Qiagen) which makes use of Boom's extraction method (Boom et al., 1998) and silica columns is widely used since it provides high sensitivity and reproducibility (Fischer et al., 1999; Fransen et al., 1998; Niubo et al., 2000). Automated extraction systems are ideally suited for the high volume requirements occurring in the routine diagnostic laboratories in South Africa. The MagNA Pure LC instrument (Roche) offers efficiency of extraction that is equivalent to the BioRobot 9604 system (Qiagen, Inc., Chatsworth, CA) (Espy et al., 2001), and was available for the purposes of this study. Nucleic acid extraction kits used with the MagNA Pure system utilize magnetic glass particles for capture and purification of nucleic acid molecules. The MagNA Pure method when compared to the NucliSens manual extraction kit (BioMérieux) requires 10 –fold smaller volumes of plasma to yield equivalent amounts of nucleic acid as the NucliSens kit (Mohammadi et al., 2003).

Real-time PCR is a relatively new technology that has been widely implemented in research and diagnostics fields. The entire process of amplification and detection occurs in one closed system with no need for post-PCR manipulations. This significantly decreases the risk of carry-over contamination with the PCR product and reduces hands-on time. Real-time PCR instruments offer high throughput, up to 384 samples per run, and the more recent developments of this technology are directed towards full automation. All of the above in combination with dramatically improved detection and quantitation of product makes real-time PCR a very attractive technology for application in the diagnostics field. In particular, a number of real-time PCR assays, both commercial and in-house, have been developed recently for detection and quantitation of viral pathogens (Candotti et al., 2004; Katsoulidou et al., 2006; Keyaerts et al., 2006; Sum et al., 2005; Swanson et al., 2006). Quantitative real-time RT-PCR using the LightCycler® technology in combination with FRET probes, LUX ™ primers and manual and automated extraction methods have been explored in this study as potential alternatives for more affordable HIV-1 viral load testing in South Africa.

2.2 Materials and Methods

An overview of methodologies for the development of an in-house HIV-1 viral load assay is depicted below.



This diagram provides a comprehensive overview of a multi-step process involved in production of ES and IC. Viral RNA was extracted using manual and automated (MagNA Pure, Roche) methods. Initially, the FRET probe based assay was developed, followed by the final version of an in-house assay using the LUX™ primer detection format.

2.2.1 HIV-1 specific primers

A previously well characterized conserved sequence within the p24 region of the HIV-1 *gag* gene (Figure 2.5) was selected as an amplification target (Bogh et al., 2001; Grankvist et al., 1996; Triques et al., 1999). Forward primer SKT145 and reverse primer SKT150 (Olfert Landt, personal communication, TIB MOLBIOL, Berlin, Germany) were manufactured by TIB MOLBIOL (Berlin, Germany) and define 128 bases region within the HIV-1 *gag* gene. The primer SKT145 represents a 9 nucleotides truncated version of a previously described primer SK145 (Bogh et al., 2001; Grankvist et al., 1996; Triques et al., 1999). The primer SKT150 partially overlaps with the primer SKCC1B (Grankvist et al., 1996; Triques et al., 1999) and is located internally to this primer.

The sequences of the primers are as follows:

SKT145 - 5' -ACA TCA AGC AGC CAT GCA AAT -3'

SKT150 - 5' -TGT CAC TTC CCC TTG GTT CTC TC - 3'

Sequences of the primers were checked for the degree of homology using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). No homology was found with sequences other than HIV-1.

2.2.2 Preparation of an External Standard (ES) for quantitation of HIV-1 RNA

2.2.2.1 Preparation of the RNA template for cloning

A plasma sample from a South African patient with a high (5 $\log_{10}$) HIV-1 viral load as estimated by the Roche Amplicor assay was used to manually extract viral RNA for template preparation (see section 2.2.4.1). Extracted HIV-1 RNA was used as a template for conventional one-step RT-PCR performed on the MyCycler™ thermal cycler (BIO-RAD Laboratories, Inc., USA). A total reaction volume of 50 µl was used containing 25.5

µl of a master mix assembled using the QuantiTect™ RT-PCR kit (Qiagen GmbH, Hilden, Germany), SKT145 and SKT150 primers at a final concentration of 0.5 µM and 20 µl of RNA. The thermal cycling conditions included an initial reverse transcriptase (RT) step performed at 50ºC for 45 minutes (min) and a hot start at 95ºC for 15 min followed by 40 cycles of amplification with a denaturation step at 95ºC for 1min, annealing at 58ºC for 30 seconds (s), and extension at 72ºC for 45 s; and a final elongation for 7 min.

The amplicons of HIV-1 generated with primers SKT145/150 were verified using conventional 2% agarose gel electrophoresis (Appendix B; see below). The RT-PCR product was diluted 1:100 in molecular grade water (MGW) provided in the QuantiTect™ RT-PCR kit. The diluted PCR product served as the template in a second round PCR performed using PCR Master Mix (Promega, Madison, WI, USA) to ensure amplification of DNA fragments possessing 3'-A overhangs for subsequent cloning. Fifty µl of PCR mix consisted of 25 µl of the reaction mix from the kit, primers SKT145 and SKT150 at the final concentration of 0.5 µM, 15 µl of PCR grade water from the kit and 5 µl of the diluted first round PCR product. Thirty cycles of PCR were performed according to the following amplification profile: initial denaturation step at 95ºC for 2 min followed by 40 cycles of amplification with a denaturation step at 95ºC for 1min, annealing at 58ºC for 30 s, and extension at 72ºC for 45 s; final elongation for 7 min. Ten microliters of amplification product was mixed with 5 µl of the gel loading dye (Fermentas UAB, Lithuania) and loaded onto a 2% agarose gel (Agarose MP, Roche Applied Science, Mannheim, Germany) prepared with 1xTAE buffer (see Appendix B) and 3 µl of ethidium bromide stock solution (10 mg/ml; Sigma-Aldrich., St. Louis, Missouri, USA) per 100 ml of gel. Agarose gel electrophoresis was performed at 100V for 1-2 hours. Size verification of the product was performed by running 10 µl of the O'GeneRuler™ 50bp DNA ladder

(Fermentas, UAB, Lithuania) along side the sample. The gel was viewed under the UV light. After product verification, the remaining PCR amplification mix was loaded onto a 1% agarose gel (~20 µl per well) and electrophoresis was performed as described above. The gel band was excised and purified using the MinElute™ Gel Extraction kit (Qiagen, GmbH Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 10µl of the elution buffer supplied in the kit.

Quantitation of the purified PCR product was performed using a PicoGreen® dsDNA quantitation kit (Molecular Probes, Eugene, USA) and the FLX 800 microplate fluorescence reader (BIO-TEK Instruments, Inc., Vermont, USA). The kit contains PicoGreen® dsDNA quantitation reagent, which is an ultrasensitive fluorescent nucleic acid stain used for quantitation of double-stranded DNA (dsDNA) in solution. The quantitation standard (λ DNA) from the kit and the PCR sample were diluted according to the protocol supplied with the kit. The high-range standard curve included the following concentrations of the λ DNA standard: 1 µg/ml, 500 ng/ml, 100 ng/ml, 20 ng/ml and the no λ DNA (blank) sample. Two dilutions of PCR product - 1:50 and 1:100 were used for quantitation. Total reaction volume (200 µl) of the diluted standards and the diluted amplification product were loaded onto the microtiter plate and fluorescence was recorded on the FLX 800 fluorimeter. The KC Junior™ microplate data analysis software (BIO-TEK Instruments, Inc., Vermont, USA) was used to generate a standard curve for quantitation of DNA. Final concentrations of the PCR product were calculated as a mean of the two values calculated by the software for both dilutions; the dilution factor was taken into account.

2.2.2.2 Cloning the external standard

The purified PCR product (section 2.2.2.1) was used as an insert for cloning into the pGEM®-T Easy Vector System (Promega, Madison, WI, USA). The high copy number pGEM®-T Easy Vector represents a convenient system for efficient cloning of PCR product. The vector is linear and possesses 3' terminal thymidine (T) at both ends. The pGEM®-T Easy Vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinant clones by blue/white screening on indicator plates. The multiple cloning site also contains restriction sites for isolation of the desired, cloned fragment by enzymatic restriction. The process of cloning included the following steps:

- Ligation of the DNA insert (PCR product) into the vector;
- Transformation of competent cells with the recombinant vector;
- Plating out the transformed competent cells on the indicator plates for blue/white screening of colonies.

All these steps were performed strictly following the protocol included in the pGEM®-T Easy Vector System (Promega, Madison, WI, USA) kit. According to the manufacturer's recommendations different "insert: vector" molar ratios were used (in particular 3:1 and 6:1). A positive control (provided in the kit) and background control (no insert DNA) was also included in the ligation experiment. Ligation reactions were left overnight at 4ºC to achieve maximum number of recombinant plasmids. Transformation of the competent *E-coli* XL1-Blue stains (Stratagene, USA) using the pGEM®-T Easy Vector ligation reactions were also performed according to the manufacturer's instructions (Promega, Madison, USA). The SOC medium required for transformation was prepared according to the recipe

provided in the kit insert (see Appendix C). One hundred µl of each transformation culture was plated onto duplicate Luria Bertani (LB)/ampicillin/IPTG/X-Gal agar plates (Appendix C). Plates were incubated overnight at 37ºC. After incubation, several white colonies from the plate with insert: vector 6:1 ratio were selected and inoculated into 10 ml of LB/Amp broth (see Appendix C) using sterile 50 ml falcon tubes and incubated overnight at 37 ºC in the Labotec (Labotec, Midrand, SA) orbital shaker (~145 rpm).

Potential clones grown in the LB/Amp broth were centrifuged (2000 rpm) on the bench top Eppendorf centrifuge 5810R (Eppendorf AG, Hamburg, Germany) for 30 min. Recombinant plasmid DNA (i.e. pGEM®-T vector with the DNA insert) was extracted using the High Pure Plasmid Isolation mini kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Recombinant plasmid DNA was eluted in 100 µl of elution buffer supplied in the kit. The DNA yield from the plasmid preparation was estimated by performing conventional spectrophotometry using a 1:100 dilution of DNA sample. Purified recombinant plasmid DNA was also assessed by conventional agarose gel electrophoresis – 2 µl of DNA was loaded onto an ethidium bromide stained 1% agarose gel and electrophoresis performed at 80 V for an hour.

2.2.2.3 Sequencing the plasmids

Three out of several different clones were selected and used for sequencing in order to determine the integrity of the insert. Two µl of purified plasmid DNA (see 2.2.2.2) from the selected three clones were used in each sequencing reaction. Two primers (Inqaba Biotech, Pretoria, SA) specific for the T7 and SP6 promoter regions of the pGEM®-T vector, were used to sequence the region in both directions. Primer sequences were as follows: T7 – 5'-TAA TAC GAC TCA CTA TAG GGC -3'; SP6 – 5'-ATT TAG GTG ACACTA TAG AAT-3'. The sequencing reaction was undertaken with the Big Dye® Terminator version 3.1 cycle sequencing kit from Applied Biosystems (Foster City CA, USA) according to the manufacturer's instructions, on the ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems). Sequence data analysis was performed using the Sequencing Analysis V3.3 programs (Applied Biosystems) and assembled using the Sequencher program version 4.1.4 (Genecodes, Ann Arbor, MI). The edited gene fragment from the cloned insert was aligned and compared with reference sequences from the HIV-1 subtype B HXB2 isolate (http://hiv-web.lanl.gov) using the Clustal X program (Jeanmougin et al., 1998; Thompson et al., 1997); http://bips.u-strasbg.fr/fr/Documentation/ClustalX).

2.2.2.4 Synthesis of the external standard RNA

After the sequence verification, clone A5 was selected, based on the high DNA concentration, as a template for *in vitro* RNA transcription. Template preparation for ES RNA synthesis included enzymatic digestion of the plasmid and purification of the linearized template.

Plasmid DNA of A5 clone was digested using *Spe*I restriction endonuclease (Roche, Applied Science, Mannheim, Germany). Fifty µl of the digestion reaction contained approximately 5 µg of plasmid DNA, 1 x SureCut buffer H (supplied with the enzyme), 2 units of *Spe*I and molecular grade water (Sigma-Aldrich, St. Louis, Missouri, USA). The reaction was incubated at 37ºC for 2 hours. The digestion product was verified by gel electrophoresis: 10 µl of the digestion reaction and 2 µl of uncut recombinant plasmid were each mixed with 5 µl of the gel loading dye and loaded onto an ethidium bromide stained 1% agarose gel, prepared with 1xTAE buffer (Appendix B). A one kilo base (kb) molecular weigh marker (Fermentas, UAB, Lithuania) was used to confirm the size of the digested plasmid. Electrophoresis was performed at 90 V for 1-2 hour. After confirmation of complete digestion the remaining reaction was incubated at 65ºC for 15 min for heat-inactivation of the *Spe*I enzyme. Electrophoresis using the remaining reaction was performed using a 1% agarose gel as described above. The gel band containing the product was excised from the gel and purified using the MinElute™ Gel Extraction kit (Qiagen, GmbH Hilden, Germany) as per manufacturer's protocol. The initial 20 µl of reaction was eluted into 10µl of elution buffer provided in the kit.

Synthesis of the ES RNA was performed by *in vitro* transcription using the RT-PCR Competitor Construction kit (Ambion, Austin, Texas, USA) and linearized, purified plasmid of the A5 clone (see section 2.2.2.4). The transcription reaction was assembled according to the manufacturer's instructions and incubated at 37ºC overnight. After completion of synthesis, template DNA was removed by DNase I treatment, supplied in the kit.

The RNeasy® Mini kit (Qiagen, GmbH Hilden, Germany) was used to terminate the transcription reaction and purify newly synthesized RNA. The procedure was performed according to the protocol supplied with the kit. RNA was eluted with 50 µl of RNase-free water from the RNeasy® Mini kit.

The quality of RNA transcript was verified using a denaturing 15% Acrylamide/8M Urea gel (Appendix B) and 5 µl of the RNA Ladder, low range (Fermentas UAB, Lithuania). Different quantities of RNA transcript: 5 µl, 10 µl and 20 µl were mixed with the equivalent volumes of the dye supplied with the RNA ladder, and loaded onto the 0.75 mm gel. Polyacrylamide gel electrophoresis (PAGE) was performed at a constant voltage of 150 V until the faster migrating dye reached the bottom of the gel (approximately 2 hours). Following electrophoresis the gel was placed on a glass plate coated with fluorescent silica, and the RNA was visualized by exposing the gel to long-wavelength UV light using a hand-held UV lamp. The full-length product was excised from the gel with a razor blade. The gel slices were transferred to a microfuge tube and submerged by brief vortexing and centrifuging in 400 µl of Probe Elution Buffer (RT-PCR Competitor Construction kit, Ambion). The tubes were incubated overnight at 37°C. Next, the acrylamide gel slice was removed from the tube and 800 µl of 100% ethanol (Merck Chemicals, Germiston, SA) was added to the Probe Elution buffer with RNA. The tubes were incubated at - 70°C for 20 min and centrifuged at 13000 x g at 4°C for 20 min using a bench top Eppendorf 5415R centrifuge (Eppendorf AG, Hamburg, Germany). The pellet was washed once with 70% ethanol. The pellets were dried at room temperature and then dissolved in 30 µl of RNase/DNase free water (Sigma-Aldrich, St. Louis, Missouri, USA). Purified ES RNA transcript was verified again using PAGE analysis.

2.2.2.5 Quantitation of in vitro transcribed ES RNA yield

The gel purified *in vitro* ES RNA transcript was quantified using a RiboGreen™ quantitation reagent (Molecular Probes, Eugene, USA), which is an ultrasensitive fluorescent nucleic acid stain for quantitating RNA in solution and includes a ribosomal RNA quantitation standard. The procedure was performed according to the manufacturer's instructions. The high-range standard curve for quantitation included ribosomal RNA standard at the following concentrations: 1 µg/ml, 500 ng/ml, 100 ng/ml, 20 ng/ml and no RNA (blank) sample. Two ES RNA dilutions were used: 1:50 and 1:100. The full reaction volume of 200µl of each sample was loaded into a microtiter plate and fluorescence was recorded using an FLX 800 fluorimeter. Contraction of a standard curve and quantitation of ES RNA concentration was performed using KC Junior™ data analysis software. ES RNA concentration values in ng/ml calculated by the software were multiplied by the dilution factor. The final concentration of ES RNA transcript was calculated as a mean of the two concentrations obtained using both 1:50 and1:100 dilutions.

The concentration of ES RNA transcript obtained from the RiboGreen quantitation assay in "ng/ml' (i.e. weight/volume units) was converted into a concentration expressed in RNA "copy per µl". Conversion was performed according to the following calculations: (Obtained from the Roche Molecular Biochemicals Technical Note, LC, 11/2000).

- MW= Molecular Weight (g/mol)

General formula for average MW of single stranded (ss) RNA is:

(Number of base) x (340 daltons/base)

- 1 mol = 6 x 10^{23} molecules (= copies)
- Concentration of nucleic acid should be converted from ng/ml into g/µl.

- The formula below was used to calculate concentration of ES RNA transcript in copies per µl:

$$\frac{6 \times 10^{23}\ \text{(copies/mol)} \times \text{concentration (g/ µl)}}{\text{MW (g/mol)}} = \text{amount (copies/µl)}$$

In order to demonstrate this type of calculation, see an example using 5 µl of ES RNA transcript loaded onto the denaturing gel and purified from the excised gel band (see 2.2.2.4):

- Concentration ES RNA transcript ("10 µl sample") estimated by RiboGreen quantitative assay was 2.7×10^{-9} g/µl
- Size of ES RNA transcript (this includes T7 promoter and a part of restriction site of pGEM -T® vector and 128 bases of cloned HIV-1 specific insert) was 192 bases.
- MW (ES RNA) = 192bases x 340daltons/base = 65280 g/mol

$$\frac{6 \times 10^{23}\ \text{(copies/mol)} \times 2.7 \times 10^{-9}\ \text{(g/ µl)}}{65280\ \text{(g/mol)}} = 2.5 \times 10^{10}\ \text{copies/µl}$$

Serially diluted ES RNA transcript was then used as a quantitation standard in quantitative real-time RT-PCR. After serial dilution of ES RNA in RNase/DNase free water each standard was spiked with MS2 phage RNA to obtain a final concentration of 8 ng/µl of the background RNA. On average, 4 to 6 external standards at different concentrations were used to construct a standard curve.

2.2.3 Preparation of an Internal Control (IC)

2.2.3.1 Construction of a template for cloning

A competitive internal control (IC) was designed to have SKT145 and SKT150 primer binding sites and an artificial sequence (scrambled HIV-1 subtype C *gag* sequence) in the region internal to these primers. Therefore, the competitive IC has the same primer binding sites, length (128 bases), and base composition as the ES. A double stranded DNA template for preparing an IC was assembled using four oligonucleotides. Sequences of these oligonucleotides were sent to MWG-Biotech AG (Germany) for synthesis.
The sequences of the oligonucleotides are as follows:

HIV (IC) S1: 5' – *CTA GAA* CAT CAA GCA GCC ATG CAA ATG TAG GAC ATG ACG AGA ATA CAT GAT AAG TAT GTG AGA GGA – 3'

HIV (IC) A1: **p** 5' – GAA TGC TCC TCT CAC ATA CTT ATC ATG TAT TCT CGT CAT GTC CTA CAT TTG CAT GGC TGC TTG ATG TT – 3'

HIV (IC) S2: **p** 5' – GCA TTC ATA CTC GAG ACA GCT CAC GTA GAG CAG GCT CGT CGC ACG AGA GAA CCA AGG GGA AGT GAC AC – 3'

HIV (IC) A2: 5' – TCG AGT GTC ACT TCC CCT TGG TTC TCT CGT GCG ACG AGC CTG CTC TAC GTG AGC TGT CTC GAG TAT – 3'

The 5' ends of the oligonucleotides A1 and S2 were phosphorylated in order to facilitate ligation. The 5' ends of oligos S1 and A2 have short overhangs – 5 nucleotides each (shown in italics). Oligos A1 and A2 correspond to the anti-sense strand, and S1 and S2 - to the sense strand. Each of the lyophilised oligonucleotides was reconstituted with RNase/DNase free water (Sigma, St Louis, Missouri, USA) to obtain the final concentration of 100 pmol/µl stock solution. Oligonucleotides S1 and S2 were initially annealed with the complementary oligonucleotides A1 and A2 respectively to form two

short, double stranded (ds) DNA fragments – S1A1 and S2A2 (Figure 2.3). Two annealing reactions were set up with oligos S1/A1 and S2/A2 diluted in RNase/DNase free water to the final concentration of 10 pmol/µl. The reactions were kept at 95ºC for 5 min and were allowed to cool down to room temperature. Next, the annealed fragments (S1A1 and S2A2) were ligated to each other using their 5' phosphorylated ends and T4 ligase (Roche Applied Science, Mannheim, Germany). The ligation reaction contained: S1A1 and S2A2 dsDNA fragments at the final concentration of 2 pmol/µl, 1Unit of T4 ligase and 1 x ligation buffer (supplied with T4 ligase kit). Ligation (Figure 2.3) was performed at 16ºC overnight.

The ligation product (138 bp DNA fragment) was used directly as a template for conventional PCR to generate amplicons suitable for cloning. Fifty µl of PCR mix consisted of 2 5 µl of ready-to use PCR Master Mix (Promega, Madison, WI, USA), primers SKT145 and SKT150 at a final concentration of 0.5 µM, 10 µl of PCR grade water from the kit and 10 µl of the ligation product. PCR was performed using the MyCycler™ instrument according to the following run profile: initial denaturation at 95ºC for 2 min, followed by 40 cycles with a denaturation step at 95ºC for 1min, annealing at 58ºC for 30 s, and extension at 72ºC for 45 s; final elongation for 7 min. Agarose gel electrophoresis for verification of amplification product, purification of the PCR product from the gel and the PicoGreen quantitative assay were performed according to the protocols described in section 2.2.2.1.

2.2.3.2 Preparation of a competitive IC RNA in vitro transcript

The PCR product (section 2.2.3.1) was used as a template for cloning into the pGEM®-T Easy Vector System (Promega, Madison, WI, USA). Cloning of the competitive IC,

isolation of recombinant plasmid DNA and estimation of DNA yield was performed according to procedures described in section 2.2.2.2. Three clones were selected for sequencing to verify the integrity of the DNA insert (see section 2.2.2.3). Edited sequences were compared to the sequence designed for a competitive IC. Clone M1 was selected as a template for further *in vitro* RNA transcription. M1 plasmid digestion with *Spe*I and purification of linearized template for RNA synthesis, *in vitro* RNA transcription using RT-PCR Competitor Construction kit (Ambion, Austin, Texas, USA), purification of IC RNA transcript and quantitation using the RiboGreen™ quantitation reagent (Molecular Probes, Eugene, USA) were performed according to the procedures described previously in sections 2.2.2.4 and 2.2.2.5.

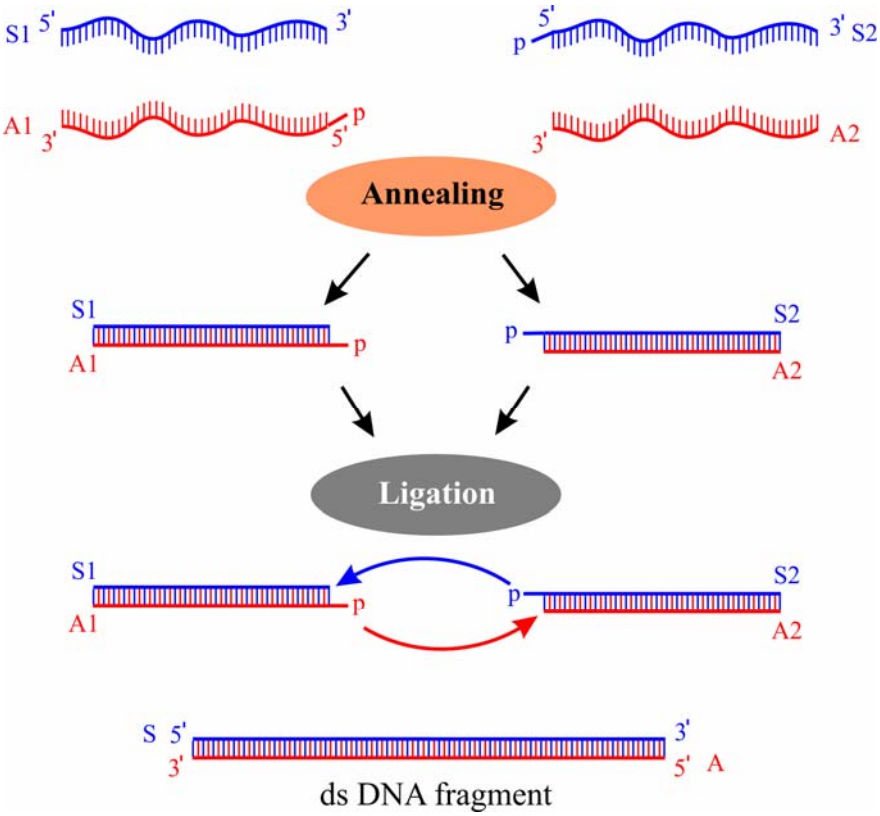


Figure 2.3: Preparation of dsDNA fragment of a competitive IC. Initial annealing step produced two dsDNA fragments: S1A1 and S2A2. Next, ligation of these fragments formed one dsDNA fragment of a competitive IC (128 bp) that contains mostly an artificial sequence (excluding the primer binding sites for SKT145/150).

2.2.3.3 Preparation of a competitive IC DNA product

M1 linearized plasmid was diluted 1:100 and 5 µl of this dilution was used as a template for conventional PCR. The reaction was performed in a 50 µl volume containing 25 µl of ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma, St Louis, Missouri, USA), 0.5 µM final concentration of each primer SKT145 and SKT150, 5 µl of the diluted M1 plasmid and 15 µl of MGW from the kit. PCR was performed using the MyCycler™ instrument according

to the following profile: initial denaturation at 95ºC for 3min and 40 amplification cycles of 95ºC for 1 min, 58ºC for 30 s and 72ºC for 45 s, with a final elongation at 72ºC for 7 min. The amplification product was verified by agarose gel electrophoresis, purified from the gel and concentration of the product was estimated using the PicoGreen quantitative assay (section 2.2.2.1). The amplification product described above represents a DNA equivalent of a competitive IC. The concentration of dsDNA competitive IC estimated using the PicoGreen quantitative assay was converted from ng/ml into copies of dsDNA per µl. Molecular weight for the dsDNA molecule was calculated as follows:

MW (dsDNA IC) = 128 bases x 660 daltons/base = 84480 g/mol

The remaining calculations of dsDNA copies/ µl were performed using the formula given in section 2.2.2.5 (Roche). This dsDNA product was used as a competitive IC in real-time RT-PCR experiments for optimization of the FRET assay.

2.2.3.4 Preparation of a non-competitive IC

The M1 linearized recombinant plasmid containing the sequence of the competitive IC (see section 2.2.3.1 and 2.2.3.2) was used as a template for generating a non-competitive IC with different primer binding sites to the ES and viral RNA. The purified PCR product of the competitive IC (section 2.2.3.1) was modified by PCR engineering to produce a non-competitive IC (Figure 2.4). This involved the replacement of SKT145 and SKT150 primer binding sites with new, different sequences, derived from the scrambled sequences of primers SKT145 and SKT150. PCR engineering was performed in two experiments using the following two sets of primers, respectively:

SK IPC 145(A): 5'- AAC CTA TCC GGA CAA TAA CGA GTA GGA CAT GAC GAG AAT-3'

SK IPC 150(A): 5'-TCC CGT CCG ATC TTT TTC TCG CTG CGA CGA GCC TGC TCT ACG-3'

SK IPC145: 5'- AAC CTA TCC GGA CAA TAA CGA-3'

SK IPC150: 5'-CTC CCG TCC GAT CTT TTT CTC GCT-3'

The designed sequence of the non-competitive IC (128 bp) amplified with primers SK IPC145 and SK IPC150 is given below:

5'- AAC CTA TCC GGA CAA TAA CGA GTA GGA CAT GAC GAG AAT ACA TGA TAA GTA TGT GAG AGG AGC ATT CAT ACT CGA GAC AGC TCA CGT AGA GCA GGC TCG TCG CAA GCG AGA AAA AGA TCG GAC GGG AG - 3'

The first set of primers (A) contains a sequence that is internal to the primers SKT145 and SKT150 with overhangs that represent new, scrambled primer sequences. The second set of primers, SK IPC145 and SK IPC150 (Inqaba Biotec, Pretoria, SA), correspond to the new, scrambled sequences that replaced the SKT145/150 primer binding sites. Two PCR experiments were performed to produce a non-competitive IC. The initial PCR was performed in a 50 µl reaction volume containing 25 µl of the ReadyMix™ reaction mix (Sigma, St Louis, Missouri, USA), 0.5 µM primers SK IPC145 (A) and SK IPC150 (A), 15 µl of MWG from the PCR kit and 5 µl of the amplification product (section 2.2.3.1). The PCR product amplified with primers SK IPC145 (A)/150(A) was verified on a 2% agarose gel.

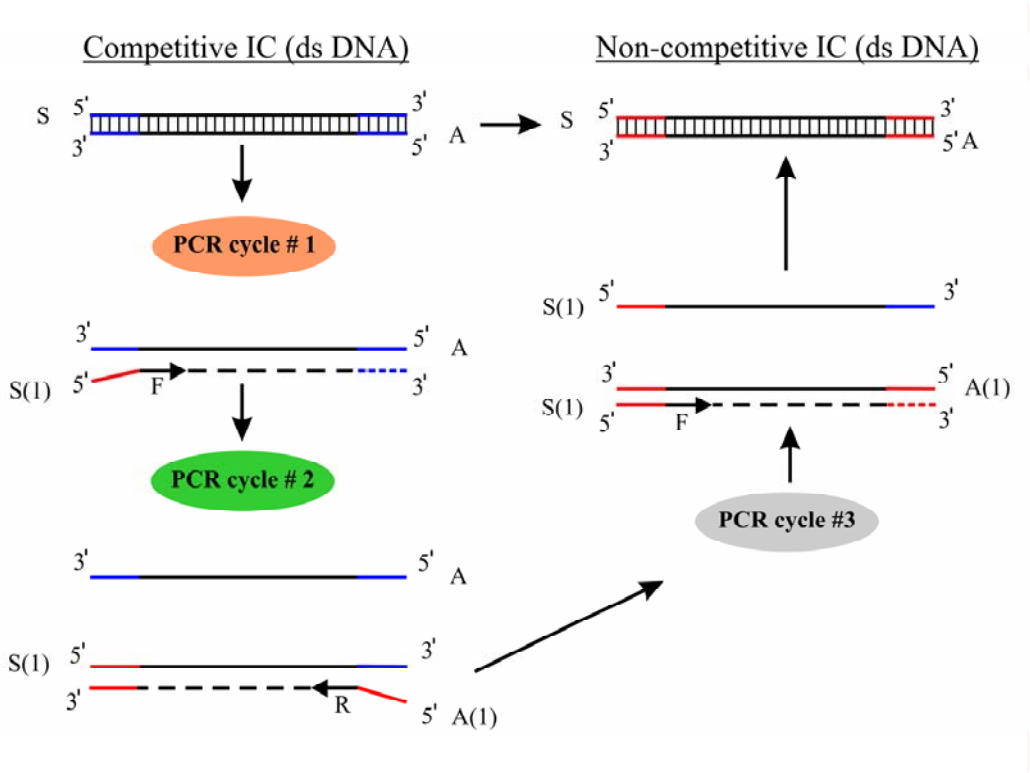


Figure 2.4: Replacement of primer binding sites in competitive IC. Primer binding sites for SKT145/150 are indicated in blue. For simplicity the process of replacing these sites is shown only for an anti-sense strand; the same process occurs simultaneously on the sense strand of dsDNA IC. The forward (F) and the reverse primers (R), internal to the primers SKT 145/150 have overhangs that correspond to the "new" primer binding sites (shown in red). Theoretically, already in PCR cycle 3, the product (S1A1) represents the first dsDNA product of a non-competitive IC with primer binding sites for SK IPC 145/150. Further PCR amplification of (S1A1) product results in accumulation of a non-competitive IC (dsDNA).

A second round PCR was then performed using 5 µl of the first amplification product, diluted 1:100 in MGW, as a template and primers SK IPC145 and SK IPC150. The amplification reaction was assembled as described above for the first round PCR. Both PCR experiments were performed on the MyCycler™ PCR instrument according to the amplification profile described in section 2.2.3.3. Verification of the amplification product by agarose gel electrophoresis, purification of the PCR product and quantitation of the non-competitive IC DNA yield using the PicoGreen quantitation reagent was performed according to the protocols described in section 2.2.2.1.

2.2.4 Isolation of HIV-1 RNA template for quantitative real-time RT-PCR

2.2.4.1 Manual extraction of viral RNA using column purification

Manual extraction of HIV-1 RNA was performed using 0.5 ml of plasma. Virus in the plasma samples was concentrated by centrifugation in a bench top Eppendorf 5415R centrifuge (Eppendorf AG, Hamburg, Germany) at 16 000 x g for 1.5 hours at 4°C. After centrifugation 360 µl of sample supernatant was discarded and the remaining 140 µl of plasma and viral pellet were used for RNA extraction using the QIAamp® Viral RNA mini kit (Qiagen, GmbH, Hilden, Germany) as per manufacturers instructions in a bench top Eppendorf 5415D mini centrifuge (Eppendorf AG, Hamburg, Germany). Column purified HIV-1 RNA was eluted in 60 µl of the elution buffer supplied with a kit.

The QIAamp® Viral RNA extraction method combines the selective binding properties of a silica-gel-based membrane/column with micro-centrifugation. According to the manufacturer's claims, this method provides high-quality and good yields of viral RNA that is free of protein, nucleases, and other contaminants and inhibitors (see QIAamp® Viral RNA Mini Kit Handbook, p.7). A maximum of twenty four plasma samples were processed simultaneously using the full sample capacity of the Eppendorf mini centrifuge.

2.2.4.2 Automated extraction of viral RNA using MagNA Pure LC instrument

Automated HIV-1 RNA extractions were performed using the MagNA Pure LC instrument (Roche Applied Systems, Mannheim, Germany) and the MagNA Pure LC Total nucleic acid isolation kit and consumables (Roche Applied Systems, Mannheim, Germany) Viral RNA was isolated from 200 µl of plasma as per manufacturer's instructions. Purified RNA

was eluted in 60 µl of elution buffer from the kit. Viral RNA samples obtained with both methods (2.2.4.1 and 2.2.4.2) were used for real-time RT-PCR immediately after isolation or stored at - 70ºC until analyzed.

2.2.4.3 Sample material

Control samples

HIV-1 RNA copy controls from the VQA laboratory (Virology Quality Assurance Laboratory, Rush-Presbyterian-St. Luke's Medical Centre, Chicago, Ill.) were used throughout assay development and evaluation. This laboratory is currently the NIH reference laboratory for viral RNA quantitation standards and protocols. The VQA RNA copy controls panel consist of five plasma samples with known concentration of virus at: $1.5x10^5$, $1.5x10^4$, $1.5x10^3$, $1.5x10^2$ RNA copies/ml and 0 copies, respectively. The VQA RNA copy controls represent cultured HIV-1 (subtype B) that has been subsequently diluted in negative human plasma. The concentration of virus in the stocks represents a mean value, which is determined using the Ultrasensitive Roche Amplicor HIV-1 Monitor™ v1.5 test and the Ultrasensitive COBAS Amplicor HIV-1 Monitor™ v1.5 test (more information is available at http://aactg.s-3.com/vqa.htm).

Another plasma sample from the HIV-1 RNA Clade performance panel (PRD201, sample ID # BBI BV-5007, clade C, Boston Biochemica Inc (BBI)) was included in this evaluation. The stock sample of this BBI panel represents a dilution of cultured HIV-1 to approximately $5x10^4$ virus particles per ml in defibrinated human plasma negative for HIV-1 RNA. The concentration of virus in the stocks was determined using Electron microscopy (EM) particle counts and the genotype was assigned by sequencing. The purchased BBI panel (www.bbii.com) was provided with a range of HIV-1 antigen and

nucleic acid test results, obtained using several commercially available HIV-1 viral load assays like Chiron HIV-1/HCV TMA Assay (GenProbe Inc., San-Diego, CA, USA), NucliSens™ HIV-1 QT (Organon Technika, Boxtel, The Netherlands) and COBAS Amplicor HIV-1 Monitor™ version 1.5 (Roche Diagnostic Systems, Branchburg, NJ, USA) amongst others.

Clinical specimens

Evaluation of the LUX assay using stored specimens (tested retrospectively) was conducted in full conformance with local ethics committee approval (Appendix D). A group of 50 known HIV-1 negative patients was used to test the specificity of the LUX assay. For these HIV seronegative plasma samples nucleic acids were extracted using the MagNA Pure LC instrument (Roche Applied Systems, Mannheim, Germany) and the MagNA Pure LC Total nucleic acid isolation kit (Roche Applied Systems, Mannheim, Germany). The extraction procedure was performed using 0.2 ml of plasma and the MagNA Pure system as described in section 2.2.4.2.

Plasma specimens were obtained from 142 randomly selected HIV-1 positive individuals from Johannesburg (South Africa) and the surrounding region. These patients were treated under the South African national roll-out program and monitored at the Johannesburg Hospital outpatient HIV clinic. The random selection of these samples was performed retrospectively, based on their HIV-1 viral load results and independent of the clinical information attached to the patient's antiretroviral (ARV) treatment status. HIV-1 viral load in these patients was routinely monitored using the standard COBAS Amplicor HIV-1 Monitor™, version 1.5 assay (Roche Molecular Diagnostics, Branchburg, NJ, USA). Viral RNA from these samples was extracted from 0.5 ml of plasma according to the manual

extraction method described in section 2.2.4.1.

The cohort of 55 patients (266 plasma samples) was obtained from the patients enrolled in the ARV treatment program supported by the Comprehensive International Program of Research on AIDS (CIPRA) and followed-up longitudinally. The CIPRA grant was provided by the National Institute of Health's Division of AIDS, funds coming from the National Institute of Allergy and Infectious Diseases. HIV–1 viral load was monitored on the CIPRA Program using both the standard and ultra-sensitive version of the Roche COBAS Amplicor assay. Samples obtained at the baseline visit (week 0) and than at the follow-up visits on weeks 4, 8, 12 and 24 were processed. All 266 plasma samples used in this study were stored at -70°C for several months before being batch processed with the LUX assay. Samples from the week 24 visit were not yet available for analysis of 9 out of the 55 patients. In this cohort HIV-1 RNA was extracted using 0.2 ml of plasma and the MagNA Pure system as described in section 2.2.4.2. In addition, negative human plasma (NHP) samples obtained from the COBAS Amplicor kits were used as controls for both the manual and automated RNA extraction methods and included in every RT-PCR run as a negative control.

2.2.5 Quantitative real-time RT-PCR using FRET probes and the LightCycler® platform (version 1.2)

Primers SKT145 and SKT150, described in section 2.2.1, were used to amplify HIV-1 RNA and the ES RNA transcript and competitive IC. The non-competitive IC was amplified using primers SK IPC145/150 (see section 2.2.3.4). Two sets of FRET hybridization probes (TIB MOLBIOL, Berlin, Germany) were used for discrimination and detection of the HIV, ES and the IC amplicons, respectively. The HIV-1 and ES amplification products were viewed in channel F2 and the IC - in channel F3 of the LightCycler®, version 1.2 instrument (Roche Applied Science, Mannheim, Germany). The sequences of primers and probes used for the FRET assay are given in Table 2.1. Sequences of the FRET probes for detection of HIV-1 RNA were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and no significant homology was found with sequences other than HIV-1.

Real-time quantitative RT-PCR was performed in a one step assay format. A total reaction volume of 31 µl contained 13 µl of master mix assembled using a QuantiTect™ Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany), 13 µl of RNA template and 2 µl of IC cDNA (1056 copies per reaction). Primers SKT145/150 were used at final concentrations of 0.5 µM and primers SK IPC145/150 were used at 0.1 µM final concentrations. Probes for detection of HIV/ES were used at final concentrations of 0.3 µM and IC probes were used at 0.1 µM. The thermal cycling profile on the LightCycler® platform comprised an RT step at 55°C for 45 min, initial activation at 95°C for 15 min, 10 cycles of 95°C for 5 s, 52°C for 30 s, 72°C for 20 s followed by 40 cycles of 95°C for 5 s, 55°C for 30 s, 72°C for 20 s. Ramping rates were 20°C/s for denaturation, 4°C/s for the annealing step and 20°C/s for

the extension.

Table 2.1: Sequences of primers and probes used in the FRET assay.

Amplification target	**Primers**	**FRET Hybridisation Probes**
HIV/ES	Primer SKT145 (Forward): 5' -ACA TCA AGC AGC CAT GCA AAT -3'	Probe labelled with a donor dye – Fluorescein* 5'- GCA TGN ACT GGA TGT AWT CTA TCC CA-Fluorescein
	Primer SKT150 (Reverse): 5' -TGT CAC TTC CCC TTG GTT CTC TC - 3'	Probe labelled with an acceptor dye - LightCycler (LC) Red 640* 5'- LC Red640 – CTG CAG CCT CYT CAT TGA TNG TRT CTT TTA - Phosphate
IC (non-competitive)	Primer SK IPC145 (Forward): 5' -AAC CTA TCC GGA CAA TAA CGA - 3'	Probe labelled with a donor dye – Fluorescein 5' – TGT GAG AGG AGC ATT CAT ACT CGA GAC A – Fluorescein
	Primer SK IPC150 (Reverse): 5' -TCC CGT CCG ATC TTT TTC TCG CT - 3'	Probe labelled with an acceptor dye - LightCycler (LC) Red 705 5' – LC Red705 – CTC ACG TAG AGC AGG CTC GTC GC - Phosphate

*Code for degenerate base positions: N = A/C/G/T; W = A/T; R = A/G; Y = C/T

Fluorescent signals were acquired during the annealing step. Melting curve analysis included 95^{o}C for 0 s, 55^{o}C for 1 min and heating up to 90^{o}C with continuous fluorescence measurement. The HIV-1 and ES amplification products were viewed using a combination of channels F2/F1 and the IC products were viewed in channels F3/F1 on the LightCycler™, instrument version 1.2 (Roche Applied Science, Mannheim, Germany).

Quantitative analysis that included construction of the standard curves was performed using the LightCycler Software version 3.5.3 (Roche Applied Science, Mannheim, Germany). This software allows for the performing of two quantitation methods – the Second Derivative Maximum and the Fit Points method. During the assay development and optimization both methods were investigated. For the preliminary evaluation of FRET assay performance only the Fit Points method was employed. Detailed description of these methods and the instructions for use are provided in the LightCycler Software version 3.5.3 manual, pages 39 to 50.

2.2.6 Quantitative real-time RT-PCR using the LUX™ primer detection format and the LightCycler® platform (version 2)

Primers SKT145, described in section 2.2.1, and the LUX™ primer SKT150 (FAM) were used in the LUX assay for amplification of HIV-1 RNA and ES RNA transcript. Modifications were made to the reverse primer SKT150 (see section 2.2.1) in order to transform it into the LUX™ detection format. According to the manufacturer's recommendations a 7 nucleotide long tail was added to the 5'-end of the primer SKT150 and a fluorescent dye FAM to the T base at the 3' end (http://www.invitrogen.com/lux; Invitrogen Corporation, Carlsbad CA, USA).

The sequences of the primers are as follows: SKT145: 5'-ACA TCA AGC AGC CAT GCA AAT -3'; SKT150 (FAM): 5'- GAG AGA ATG TCA CTT CCC CTT GGT TCT CT(FAM)C -3'. RT-PCR product amplified with primers SKT145 and SKT150 (FAM) was viewed in channel 530 nm of the LightCycler® instrument, version 2 (Roche Applied Science, Mannheim, Germany). The LUX™ primer SKT150 (FAM) was analyzed for sequence homology using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

The non-competitive IC was amplified using the forward primer SK IPC145 (see section 2.2.3.4) and the reverse LUX™ primer SK IPC150. Primer SK IPC150 has a 7 nucleotide long tail and a fluorescent tag JOE (Invitrogen Corporation, Carlsbad CA, USA) at the T base of the 3' end. The sequences of primers are as follows: SK IPC145: 5' -AAC CTA TCC GGA CAA TAA CGA - 3'; SK IPC150 (JOE): 5'- AGC GAG ATC CCG TCC GAT CTT TTT C (JOE) TC GCT-3'. The amplicons of the non-competitive IC generated with primers SK IPC145 and SK IPC150 (JOE) were viewed in channel 560 nm of the LightCycler® instrument, version 2. Inhibition of PCR is determined by the complete absence of amplification of the IC above the background levels of fluorescence or by a significant shift in the amplification curve of at least 5 threshold cycles (C_T) or more.

One step RT-PCR was performed in a 50 µl reaction volume containing 25 µl of master mix provided in the QuantiTect™ Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany), 0.5 µM of each primer, 2 U of heat labile uracil – DNA – glycosylase (HL-UNG; Roche Applied Science, Mannheim, Germany) and 22 µl of RNA template. The duplicate reactions containing IC were assembled using 25 µl of the reaction mix from a QuantiTect™ Probe RT-PCR kit, 0.5 µM primers SK IPC145 and SK IPC150, 2 U of HL-UNG, 2.5 µl of IC DNA ($2x10^5$ to $2x10^6$ copies/reaction) and 20 µl of RNA template.

The thermal cycling profile on the LightCycler® version 2 (Roche Applied Science, Mannheim, Germany) platform comprised an RT (reverse transcription) step at 55°C for 50 min, initial activation at 95°C for 15 min, 10 cycles of 95°C for 5 s, 52°C for 15 s, 72°C for 20 s, 77ºC for 3 s followed by 50 cycles of 95°C for 5 s, 55°C for 15 s, 72°C for 20 s and 77ºC. Ramping rates were 20°C/s for all the steps of the PCR cycle. Fluorescent signals were acquired during the additional heating step (77ºC for 3 s) in order to denature non-

specific product, i.e. primer dimers. Melting curve analysis included 95°C for 0 s, 50°C for 1 min and heating up to 90°C with continuous fluorescence measurement. This RT-PCR profile was used for a preliminary evaluation of the LUX assay (Rekhviashvili et al., 2006).

A slightly modified protocol to increase the stringency of amplification was used for further evaluation with clinical specimens. The modified real-time RT-PCR profile includes: RT step at 50°C for 50 min, initial activation at 95°C for 15 min, 10 cycles of 95°C for 5 s, 58°C for 25 s, 72°C for 20 s, 78ºC for 4 s followed by 50 cycles of 95°C for 5 s, 60°C for 30 s, 72°C for 20 s and 78ºC for 4 s. Ramping rates were 20°C/s and the fluorescent signal was acquired during the fourth heating step at the end of each PCR cycle - at 78°C for 4 s. Melting curve analysis was: 95°C for 0 s, 50°C for 1 min and heating up to 90°C with continuous fluorescence measurement (Rekhviashvili et al., submitted).

For the optimization of the LUX assay, quantitation was performed using a Fit points method and an Automated method provided by the LightCycler® (version 2) Software version 4 (Roche Applied Science, Mannheim, Germany). Evaluation of the LUX assay was performed using an Automated (user non-influenced) method for quantitative analysis. Characterizations of both quantitation methods and the recommendations for use are available in the LightCycler® (version 2) Operator's Manual (version 1.2), pages 95-110. For the preliminary evaluation of the LUX assay using ES RNA transcripts, the concentration of standards were entered into the software (version 4) in copies/ml. ES values in copies/ml were calculated based on the known input concentration of each standard and only the "real-time" standard curves were used (i.e. non-imported). For evaluation of the LUX assay using clinical specimens two to three external standards were

included in each RT-PCR run in order to import the best suitable standard curve into the run. Concentrations of standards were entered into the software in copies/µl and thus HIV-1 viral load values for the plasma samples were obtained in the same units. Since different extraction methods were used for patient samples, final viral load values were re-calculated taking into account starting volume of plasma and RNA elution volume, and the corresponding values in copies/ml were obtained. For example: 5000 copies/µl x 60 µl/ 0.2 ml = 150 000 copies/ml. In this example, 5000 copies/µl is the viral load value obtained with the LUX assay (an arbitrary value), 60 µl is the elution volume and 0.2 ml is the starting volume of plasma.

2.2.7 Viral load quantitation using COBAS Amplicor HIV-1 Monitor™, version 1.5 assay

The Roche AmpliPrep/Amplicor system was used as the reference methodology for all comparative evaluations conducted in this study (see section 1.2 for description of the assay). HIV-1 RNA extraction was conducted using the AmpliPrep automated specimen preparation system followed by amplification and detection using the Roche COBAS Amplicor HIV-1 Monitor™ 1.5 assay (Roche Molecular Systems, Branchburg, N.J.) All assays were performed strictly according to the manufacturer's instructions. For the standard procedure 0.35 ml of plasma sample, and for the ultra sensitive version of the assay - ,0.7 ml of plasma was used.

2.2.8 Statistical analysis

Statistical analysis for the FRET assay, in particular determination of the linearity of the dynamic range, was performed using the Stata™ 7 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA). Both versions (1.2 and 2.0) of the LightCycler®

system evaluated in this study provide standard deviation (SD) and mean concentration values for the replicate testing of the samples. SD and $\log_{10}$ transformed mean concentration values obtained for both the FRET and the LUX assays were entered into the Microsoft Excel program and used to calculate the coefficient of variation (CV) for each assay, respectively.

The main statistical analysis for clinical evaluation of the LUX assay was performed in Stata™ 9.1 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA) using log (base 10) transformed viral load values. Histograms and box plots were used to assess distributions of $\log_{10}$ viral loads observed for both LUX and COBAS Amplicor assays. Agreement between the two assays was assessed by means of the intra-class correlation coefficient (ICC) estimated via a one-way analysis of variance (Snedecor and Cochran, 1989) as implemented in Stata™ 9.1. In addition a Bland-Altman plot (Bland and Altman, 1986), a well recognized method for measuring agreement between two assays measured on a continuous scale, was generated. Differences between the $\log_{10}$ values of the data pairs are graphically represented on the vertical axis against the mean of the $\log_{10}$ values of the data pairs (mean $\log_{10}$ copies/ml) on the horizontal axis. The mean $\log_{10}$ paired difference (the bias) and the limits of agreement (mean ± 2SD, standard deviation) are indicated on the plots. To assess for changes in viral load obtained with the LUX assay and the COBAS Amplicor test over patient's visits, schematic (box and whisker) plots were used. A mixed-effects model could not be used to analyze trends over visits because once treatment commenced, viral load values on both assays dropped to undetectable levels, thus showing little variability over subsequent visits.

2.3 Results

2.3.1 Optimisation and preliminary evaluation of quantitative real-time RT-PCR using FRET hybridization probes and the LightCycler® platform (version 1.2)

DNA template for *in vitro* synthesis of an ES RNA was successfully cloned, sequenced and analyzed. The nucleotide sequence was aligned with a reference *gag* sequence of HIV-1 subtype B HXB2 isolate (Figure 2.5), confirming the correct region had been cloned. Therefore, the ES RNA *in vitro* transcript (192 bases) contains a sequence of 128 bases that corresponds to the fragment within the p24 region of HIV-1 *gag*. Phylogenetic analysis revealed that the cloned fragment was subtype C specific in this region of p24 (results not shown). Good quality and highly concentrated ES RNA (Figure 2.6) stock solution was diluted to 10^9 and 10^8 copies/µl working stocks and kept at -70ºC. Serial 1:10 dilutions of ES RNA covering the concentration range of 10^3 – 10^7copies/ml were used for optimization of a new quantitative real-time RT-PCR for HIV-1 viral load quantitation. ES RNA samples of each concentration (10^3, 10^4, 10^5, 10^6 and 10^7 copies/ml) were run in replicates in every RT-PCR experiment. One or two sets of ES RNA replicate samples (10^3 – 10^7 copies/ml) were entered into the LightCycler® software version 3.5 as quantitation standards to generate a standard curve, and the remaining replicate ES RNA samples were entered as "unknown" samples.

Initial assay development was performed on the LightCycler® platform (version 1.2) using FRET hybridization probes as a detection format. Several parameters of the quantitative real-time RT-PCR had to be optimized. Optimization of the RT-PCR profile showed that a longer incubation time (50 min) for the RT step and ten cycles of initial amplification with

a lower annealing temperature increased the sensitivity of quantitation. Different concentrations of SKT 145/150 primers (0.25 μM, 0.5 μM and 0.75 μM) and FRET hybridization probes (0.2 μM, 0.3 μM, and 0.4 μM) were titrated for the ES RNA range of $10^3 – 10^7$ copies/ml. The experiments showed that a final concentration of primers at 0.5 μM and final concentration of each of the hybridization probes at 0.3 μM were found to be optimal for the wide range of ES RNA concentrations used. The addition of MS2 phage RNA as a stabilizing background nucleic acid to each of the ES RNA dilutions ($10^3 – 10^7$ copies/ml) significantly improved sensitivity, accuracy and reproducibility of a standard curve based quantitation (data not shown). Numerous RT-PCR runs were performed to identify the most optimal concentration of an RNA transcript of the competitive IC. These experiments revealed that approximately a 1000 copies/ml of IC RNA can be co-amplified with a range of ES RNA from 10^4 -10^6 copies/ml. The addition of IC RNA to RT-PCR reactions reduced sensitivity and accuracy of quantitation (data not shown).

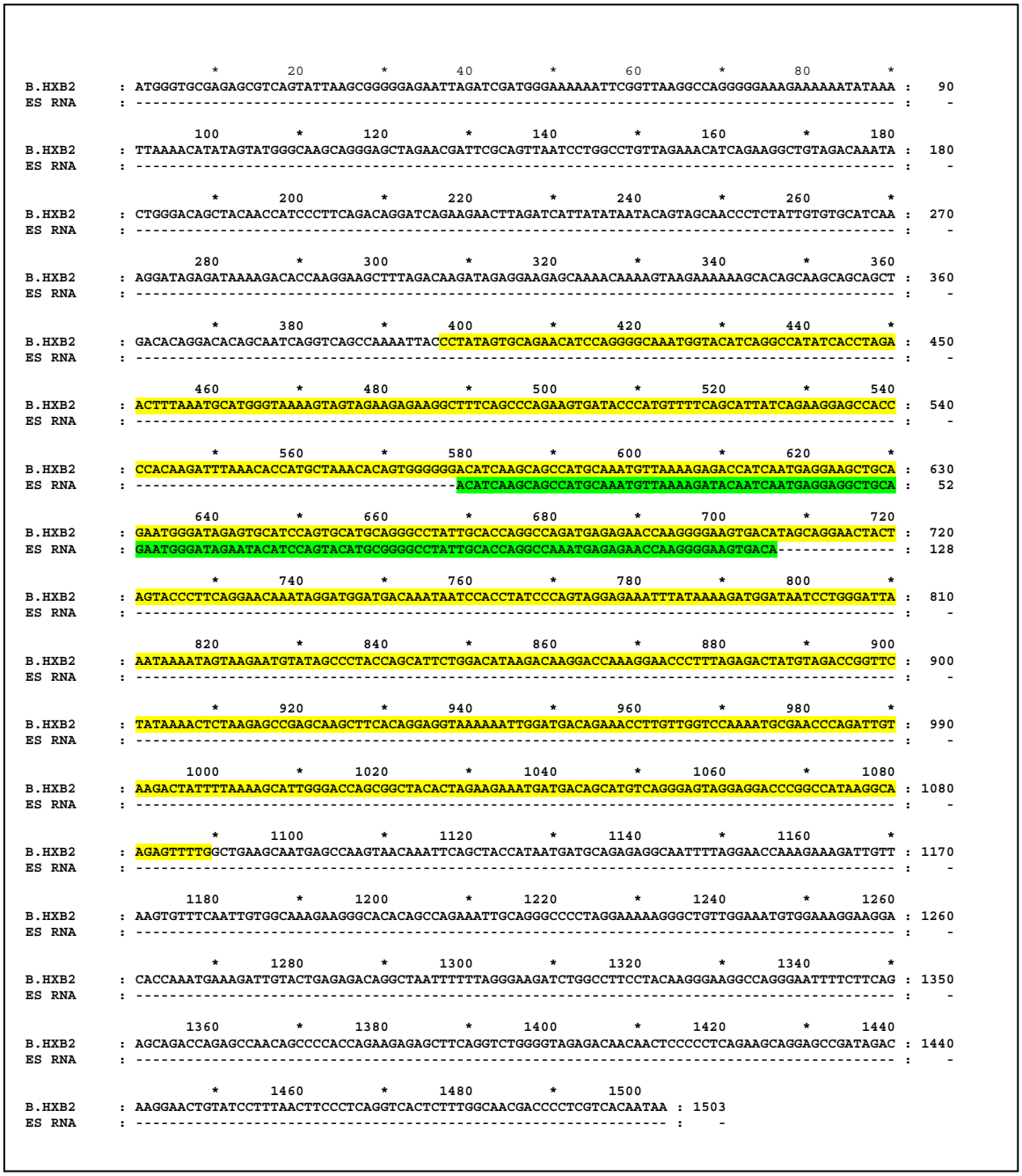


Figure 2.5: Nucleotide sequence alignment of gag (p55) from the HIV-1 subtype B HXB2 isolate with the ES RNA synthetic fragment. The p24 region is highlighted in yellow, and the ES RNA synthetic fragment is shown in green.

The attempts to use DNA IC were even less successful. Next, multiple experiments were performed using DNA of a non-competitive IC, where concentrations of a non-competitive IC

DNA and primers SK IPC145/150 were titrated simultaneously. The most optimal combination was represented by 1056 copies of a non-competitive IC DNA per reaction amplified using 0.1 μM primers (Figure 2.7).

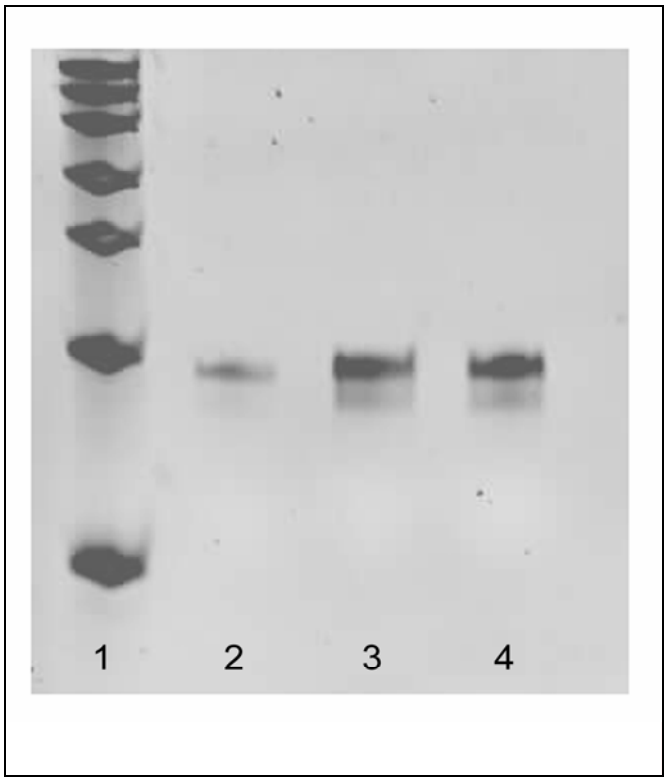


Figure 2.6: Denaturing polyacrylamide gel electrophoresis for verification of purified ES RNA transcript. Lane 1 - RNA molecular weight marker (100 bases); lanes 2, 3, 4 - show 5, 10 and 20 μl of ES RNA transcript (~ 192 bases) loaded onto the gel, respectively.

Preliminary evaluation of the developed quantitative real-time RT-PCR (FRET assay) was performed using ES RNA transcript, VQA copy controls and the BBI proficiency panel. In order to assess reproducibility of the FRET assay ten - fold serial dilutions of ES RNA covering the range from $1.5x10^3$ to $1.5x10^7$ copies/ml were used in five RT-PCR

experiments performed on different days. Each of five standards was spiked with non-competitive IC (1056 copies/reaction) and tested in six replicates within each run.

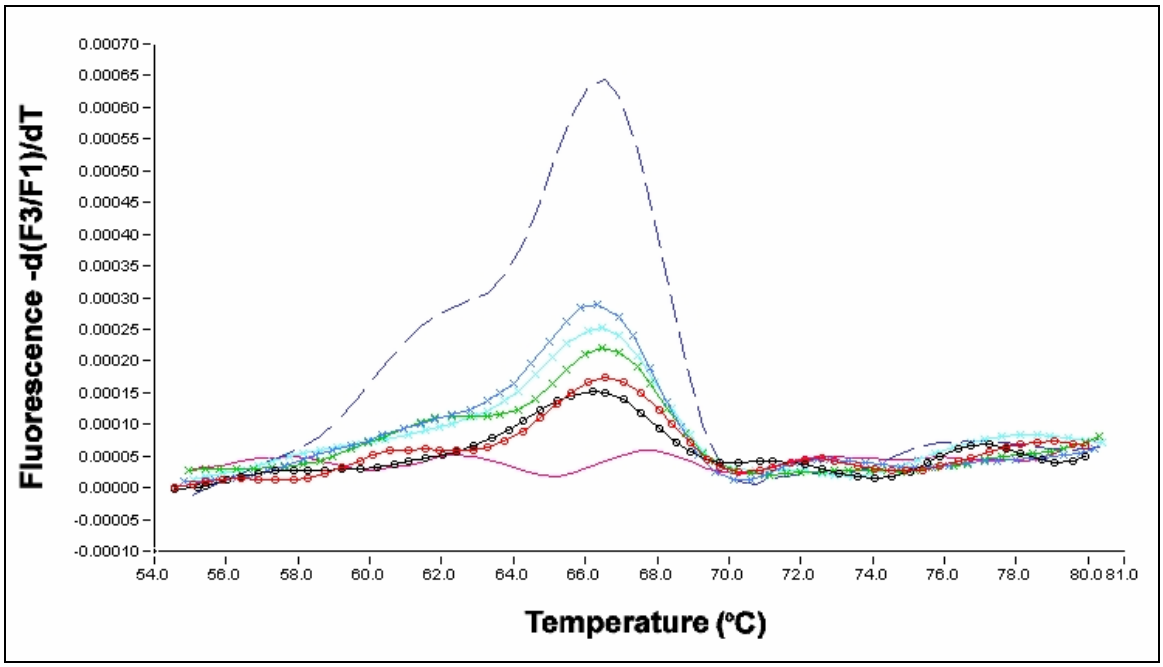


Figure 2.7: Post-PCR melting curve analyses for titration of a non-competitive IC.
Melting curve profiles show amplification of a non- competitive internal control (IC) DNA spiked into ES RNA samples. Key: sample containing IC only (- -); ES RNA dilutions from 1.5 x 10^3 - 1.5 x 10^5 copies/ml (**x**), ES RNA dilutions at the concentration of 1.5 x 10^6 and 1.5 x 10^7 copies/ml (o); no IC sample (-) shows the absence of a melting peak.

The coefficient of variation (CV) for reproducibility of the FRET assay was calculated based on the mean of the calculated concentration values and the corresponding standard deviation (SD). Intra-assay variability CV for the FRET assay was determined for the replicated standards on each particular day, and found to range from 23% CV to 43, 3% CV for 1.5x10^7 and 1.5x10^4 copies/ml of ES RNA, respectively. Inter-assay variability of the FRET assay was 23, 1% CV and 7, 4% CV for 1.5x10^7 and 1.5x10^4 copies/ml of ES

RNA, respectively. Analytical sensitivity of the FRET assay was estimated based on the repeated runs (5 experiments with 6 replicates of each standard). The standard of $1.5x10^3$copies/ml could not be quantified reproducibly within each run (Figure 2.8). Reproducible quantitation (100%) was observed at the standard of $1.5x10^4$copies/ml that defined the lower limit of quantitation (LLQ) of the FRET assay.

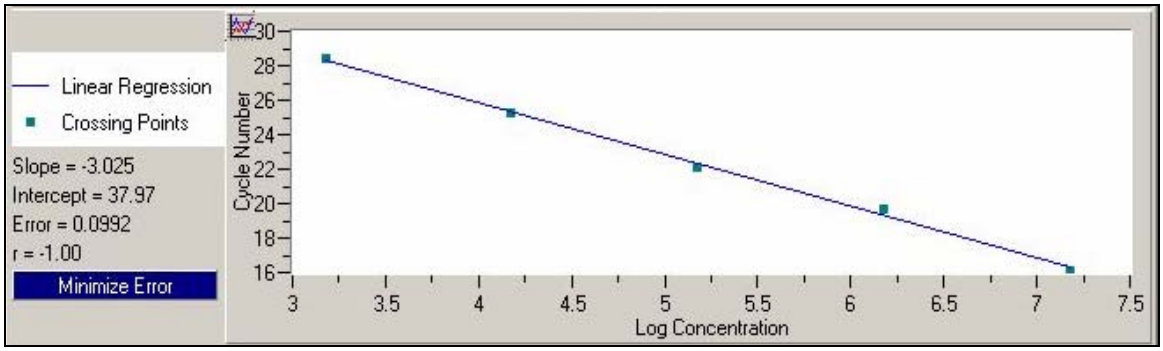


Figure 2.8: Standard curve generated using serial dilutions of ES RNA transcript. Quantitation range covers $1.5x10^3$ to $1.5x10^7$ copies/ml of ES RNA transcript. The standard curve was generated using the "fit points" analysis (software version 3.5) on the LightCycler® instrument (version 1.2).

Linearity of the dynamic range for the FRET assay was established using between-run mean values of calculated ES RNA copy numbers obtained from the repeated runs (Figure 2.9). The coefficient of linear regression $R^2 = 0.98$ was obtained for the quantitation range of $1.5x10^4$ to $1.5x10^7$ copies/ml of ES RNA.

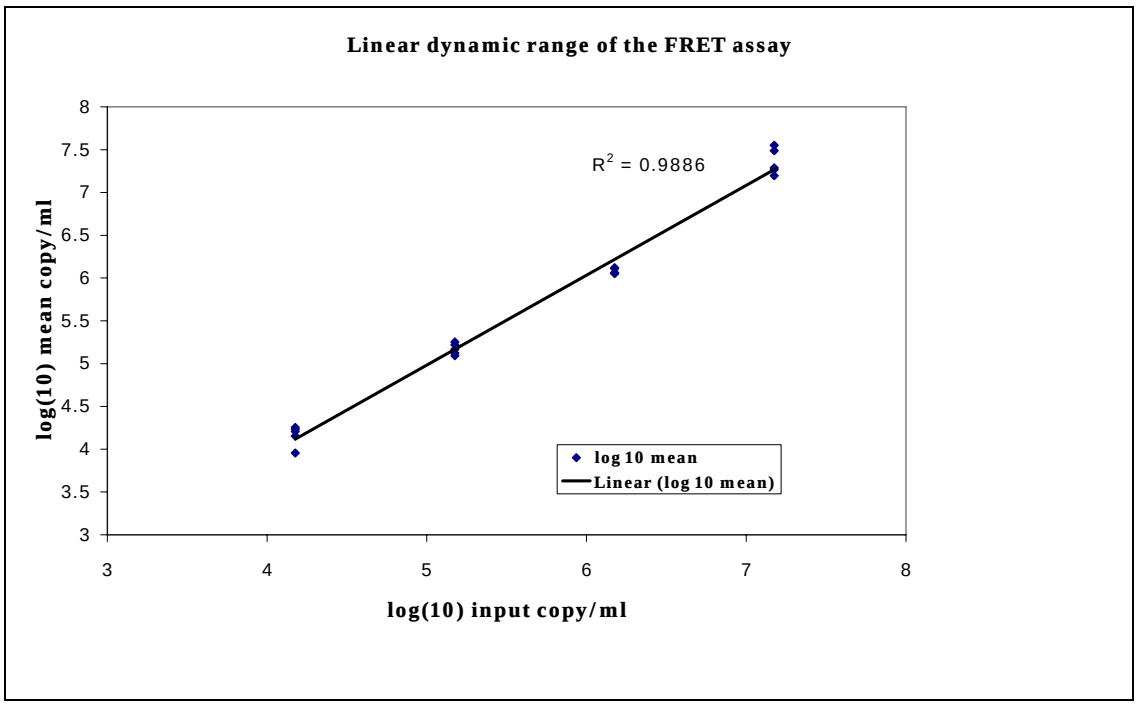


Figure 2.9: Linear regression analyses for the dynamic range of the FRET assay.
Log_{10} transformed mean values of experimentally established ES RNA copy numbers obtained from the repeated FRET assays (5 repeat runs with six replicates of each standard from $1.5x10^4$ to $1.5x10^7$ copies/ml of ES RNA) are plotted on the Y-axis. Log transformed values of the expected (input) concentrations for the same standards are plotted on the X-axis.

In order to verify the in-house external quantitation standard (ES RNA), viral RNA extracted from the VQA copy controls was used. No quantitation of HIV-1 RNA was obtained in all four VQA samples using the FRET assay. However, conventional gel electrophoresis revealed the presence of HIV specific amplification product in the VQA samples with viral load values of $1.5 x10^4$ and $1.5 x10^5$ copies/ml (data not shown). Therefore, the unsuccessful quantitation observed with the VQA copy controls was attributed to the insufficient detection of amplicons with the FRET hybridization probes.

Verification of the external quantitation standard (ES RNA) and preliminary evaluation of the FRET assay were performed using the BBI proficiency panel. Viral RNA extracted from the stock sample was tested in triplicate using the FRET assay. The quantitative analysis of this experiment was performed using a standard curve ($1.5x10^3$ to $1.5x10^7$ copies/ml of ES RNA) generated in the same run as well as a standard curve imported from a previous run. Real-time quantitation of the triplicate sample showed a mean of 38 403 copies/ml. Quantitation using an imported standard curve revealed a mean of 42 267 copies/ml. An average viral load value obtained using both real-time and an imported standard curve was 40 335 copies/ml, corresponding to a 4.6 $\log_{10}$ value. According to the package insert for the BBI panel the COBAS Amplicor version 1.5 (Roche) test estimated viral load in the BBI sample we used at 20 000 copies/ml (4.3 $\log_{10}$) and the NucliSens™ HIV-1 QT (Organon Technika) at 10 000 copies/ml (4 $\log_{10}$). Therefore, the FRET assay estimated viral loads of the BBI HIV-1 proficiency panel in the same $\log_{10}$ range of concentration as the COBAS Amplicor and NucliSens™ assays. An absolute difference between the $\log_{10}$ values obtained with FRET assay and the COBAS Amplicor assay is within absolute 0.3 $\log_{10}$ difference.

2.3.2 Optimisation and preliminary evaluation of quantitative real-time RT-PCR using LUX™ primer and the LightCycler® platform (version 2)

A quantitative real-time RT-PCR assay for determination of HIV-1 viral load was initially developed using FRET hybridization probes as a detection format and version 1.2 of the LightCycler® platform. Subsequently, the developed assay was transferred onto a newer, more advanced version of the LightCycler® instrument (version 2) once it became available. The latest, glass capillary based LightCycler® platform (version 2) allowed

modifications and further improvement of the in-house viral load assay. In particular, FRET probes were replaced with a LUX™ primer for amplification and simultaneous real-time detection of amplicons. The real-time RT-PCR profile was adapted to the LUX™ primer detection format by the addition of a forth segment at the end of each amplification cycle. The additional segment was performed at 77ºC for 3 s and during this time a fluorescent signal from the LUX™ primer was acquired. A larger reaction volume (50 µl) increased template RNA input volume up to 22 µl. Optimisation of the quantitative real-time RT-PCR using the LUX™ primer and the LightCycler® platform (version 2) was performed using serial dilutions of the ES RNA transcript covering a quantitation range of $10^2 – 10^6$ copies/ml. Amplification products of ES RNA and VQA copy controls generated with the SKT150 LUX primer and modified reaction conditions were verified using agarose gel electrophoresis (Figure 2.10). Numerous experiments were performed in an attempt to determine the optimal concentrations of a non-competitive IC DNA and SK IPC 145/150 primers. These experiments showed that the minimal concentration of IC and the primers, which is sufficient for co-amplification with the higher copy numbers of ES RNA, inhibits (out competes) the lower concentration standards. Thus, to improve sensitivity of the viral load assay and achieve better accuracy of quantitation over a wide dynamic range, the non-competitive IC was not added to the external quantitation standards (ES RNA transcript) and "unknown" samples. For the LUX assay design it was decided to add IC only to a duplicate test of an "unknown" sample that was not used for quantitation of HIV-1 RNA copies. During evaluation of the LUX assay the duplicate tests were used solely to control for PCR inhibition.

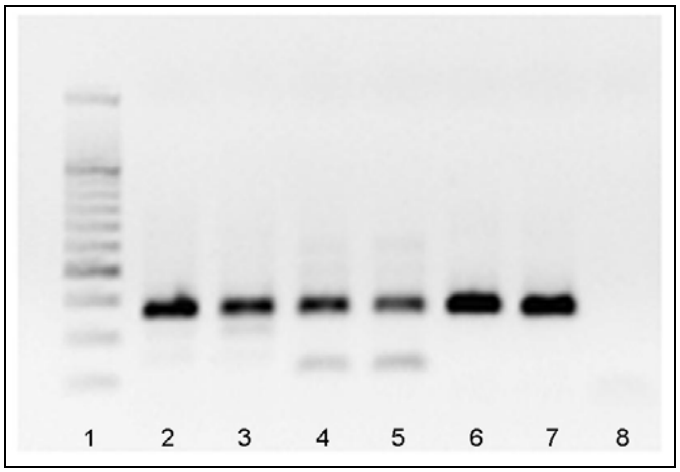


Figure 2.10: Conventional 3% agarose gel electrophoresis for confirmation of RT-PCR product obtained using the LUX assay. Lane 1- 50 bp DNA ladder; lanes 2 and 3 – ES RNA samples at the concentrations of 4 $x10^6$ and 4 $x10^5$ copies/ml respectively; lanes 4 and 5 – VQA samples at 1500 and 15 000 copies/ml respectively; lanes 6 and 7 - VQA samples at 150 000 copies/ml (tested in duplicates); lane 8 – negative human plasma (NHP) sample showing no amplification product (negative control).

Preliminary evaluation of the LUX assay was performed using serial dilutions of the ES RNA transcript. The following performance characteristics were investigated: analytical sensitivity, reproducibility and linearity of the dynamic range. Sensitivity of the LUX assay was established using serial dilutions of ES RNA transcript covering a quantitation range from 40 to $4x10^4$copies/ml. ES RNA samples at each concentration were tested in five replicates within a run. Concentration of each standard in copies/ml was calculated based on the input copies of ES RNA transcript (i.e. 22 µl of ES RNA in 50 µl of RT-PCR reaction). Sensitivity was 60% at 8 x 10^1copies/ml and 100% at $4x10^2$copies/ml. Therefore, the LLQ of the LUX assay is set at 400 copies/ml.

The reproducibility of the assay was determined as within run (intra-assay) and between run (inter-assay) variability. Repeated tests for reproducibility of the LUX assay were

performed on four different days and each of the six quantitation standards was repeated four times within a run. Similarly to the LLQ study, concentration of ES RNA transcript in copies/ml was established based on ES RNA input concentration. For example, 22 µl of the quantitation standard (ES RNA) at a starting concentration of 1 x 10^4 copies/ µl correspond to a concentration of 4 x 10^6 copies /ml when a 50 µl reaction volume is used. Concentrations of the LUX assay standards used to establish reproducibility were as follows: $4x10^{2,}$ $8x10^2$, $4x10^3$, $4x10^4$, $4x10^5$ and $4x10^6$ copies/ml (Figure 2.11). The mean value of the experimentally obtained concentrations for six replicates of each standard was used to assess intra-assay (within run) variability. The SD for the corresponding mean values of concentrations were calculated by the software (version 4) of the LightCycler® instrument (v 2). For inter-assay variability mean concentrations and SD(s) obtained from 4 repeated runs were used to determine overall mean values for concentrations and SD(s), respectively. The CV for intra- and inter-assay variability of the LUX assay was calculated based on the log transformed mean concentration values and the corresponding SD values: (SD/Log Mean conc.) x 100% = CV(%) . The intra- and inter-assay variability (CV %), reflecting the reproducibility of the LUX assay, is shown in Table 2.2.

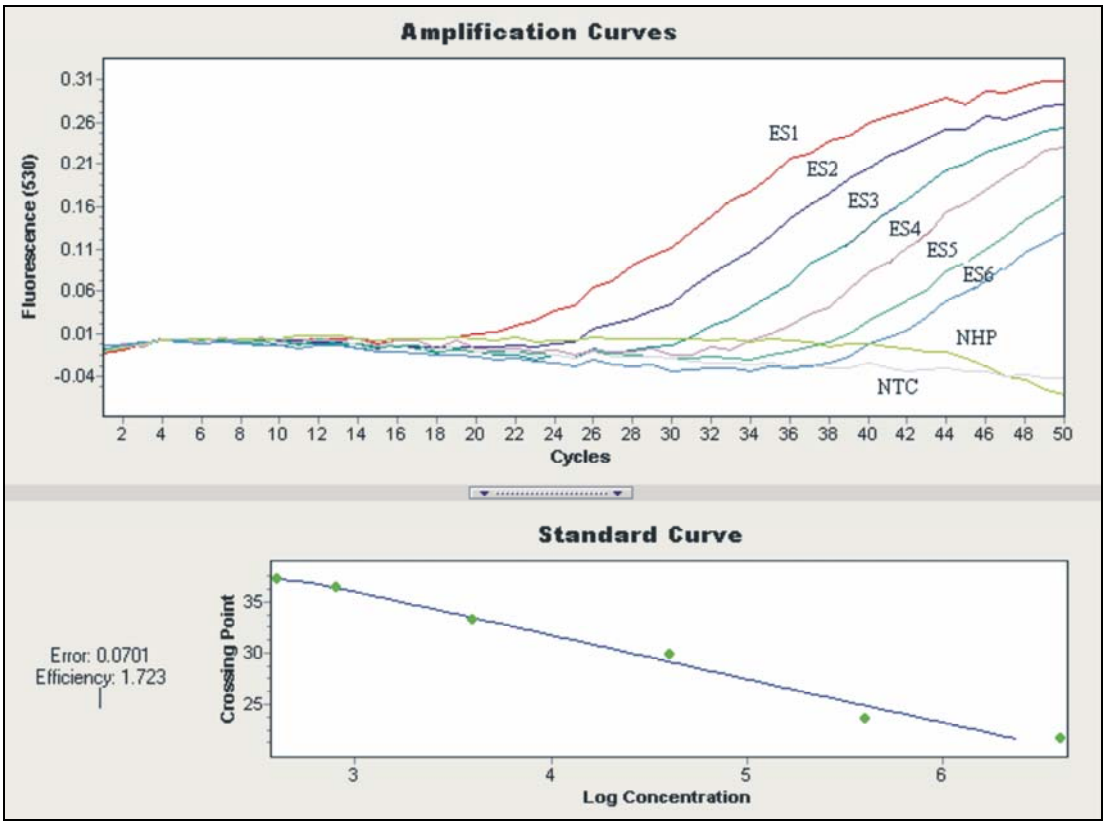


Figure 2.11: Amplification curves and an external standard curve generated using the LUX assay (Adapted from Rekhviashvili et al., 2006). The amplification curves reflect the differences between serially diluted ES RNA samples, which are used to generate the external standard curve for quantitation of HIV-1 RNA copies. Concentrations of the ES1 to ES6 RNA standards were $4x10^6$, $4x10^5$, $4x10^4$, $4x10^3$, $8x10^2$ and $4x10^2$copies/ml, respectively. The negative control (NHP) and no template control (NTC) samples are indicated. The standard curve was obtained with the Automated method for quantitation on the LightCycler® instrument, version 2.

Table 2.2: Intra- and Inter-assay variability of the LUX assay using log_{10} transformed concentrations (ES RNA copies/ml)

The four middle columns (replicate tests 1,2,3,4) represent the intra-assay variability for each replicate/day test and include the mean concentration values of four replicates of each standard and the intra-variability CV. The last column represents the inter-assay variability (shown by the inter-variability CV) that summarizes the results over four replicate experiments.

Input Concentration (log_{10})	Replicate Test 1*		Replicate Test 2*		Replicate Test 3*		Replicate Test 4*		Replicate Tests 1-4	
	Mean concentration (log_{10})	CV%	Mean Concentration (log_{10})	CV%	Mean Concentration (log_{10})	CV%	Mean concentration (log_{10})	CV%	Mean Concentration (log_{10})	CV%
6.6	6.55	0.45	6.62	0.20	6.61	0.06	6.37	2.50	6.55	0.56
5.6	5.74	1.80	5.60	0.13	5.43	2.20	5.84	2.90	5.68	0.97
4.6	4.32	4.70	4.70	1.50	4.86	4.08	4.42	2.70	4.63	0.43
3.6	3.80	3.70	3.57	0.70	3.45	3.20	3.61	0.20	3.61	0.28
2.9	2.72	4.80	2.90	0.20	2.88	0.50	2.91	0.30	2.91	0.29
2.6	2.78	4.60	2.64	3.90	2.60	0.06	2.75	3.85	2.70	2.60

*Average of four replicates. (Table adapted from Rekhviashvili et al., 2006).

Overall variability of the LUX assay was studied in more detail. Log transformed concentration values obtained for four replicates of each standard over four different experiments were used. A plot is represented in Figure 2.12 to illustrate any data points that show a difference >0.5 $\log_{10}$ between the input standard and calculated concentration values using the log transformed values. The greatest variability was found at 2.6 $\log_{10}$ and less at 5.6 $\log_{10}$ of ES RNA concentrations. These data reflects the results shown in Table 1 where three replicate runs out of four have higher intra-assay variability CV for 2.6 $\log_{10}$ and two out of four replicate runs have an increased intra-assay variability CV for 5.6 $\log_{10}$.

Linearity of the dynamic range of the LUX assays was established based on the mean values of ES RNA concentration range ($4x10^2 – 4x10^6$copies/ml) obtained from four repeated experiments performed for reproducibility study. These values were plotted against the input copy number of ES RNA in a log-log scale. Coefficients of linear regression (R^2) of the dynamic range from $4x10^2$ copies/ml to $4x10^6$ copies/ml was R^2 = 0.99. This indicates that the LUX assay is linear over its quantitation range.

Since this viral load assay uses ES RNA for quantitation of the wild type HIV-1 RNA copies it is essential that the efficiencies of amplification of the HIV-1 *gag* region and ES RNA are comparable. RT-PCR efficiencies of ES RNA and HIV-1 were compared for the LUX assay sing serial dilutions of ES RNA and the VQA RNA copy controls. In the experiment all five VQA samples were tested in triplicate. ES RNA samples covering the dynamic range of $1.5x10^2 – 1.5x10^5$copies/ml were also included in the RT-PCR run. In order to compare RT-PCR efficiencies VQA samples were first entered into the LightCycler® software as standards and a standard curve was generated. The software

(version 4) then calculated a PCR efficiency value for every standard curve (see the LightCycler® 2.0 Instrument Operator's Manual, Version 4, page 96). RT-PCR efficiency of HIV-1 amplification (VQA copy controls) over 3 $\log_{10}$ of concentration from $1.5x10^3$ to $1.5x10^5$copies/ml was 1.9. The ES RNA amplification efficiency over the corresponding dynamic range was 2.2 (data not shown). Therefore, RT-PCR efficiencies of ES RNA and HIV-1 are comparable.

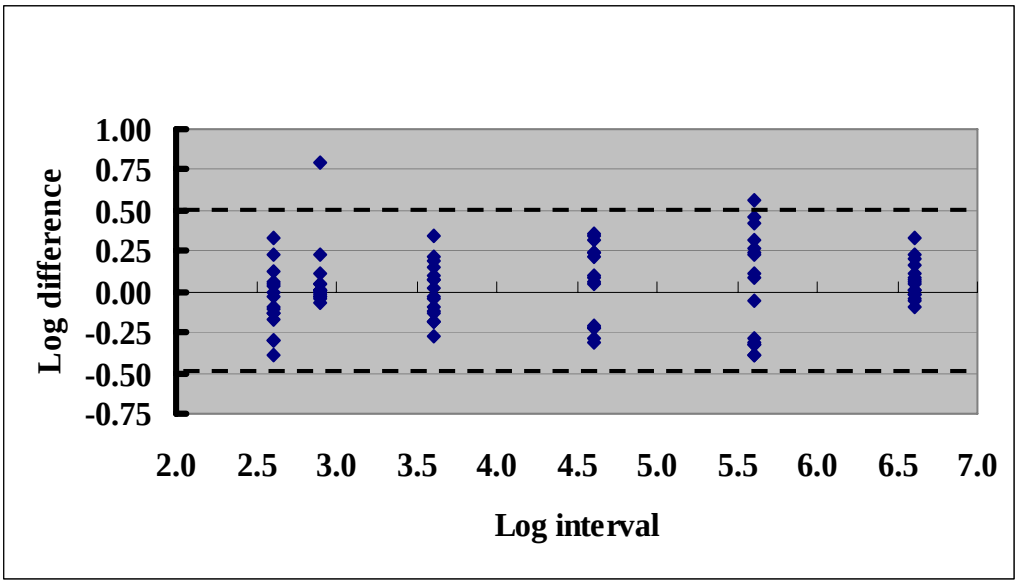


Figure 2.12: Differences in log values between input and calculated concentrations of ES RNA using the LUX assay. (Adapted from Rekhviashvili et al., 2006). Intra- and inter-assay variability was studied in four replicate runs and the standards of every concentration ($4x10^2$ - $4x10^6$copies/ml) were repeated four times within a run (n = 4 x 4). Each point in the graph represents the log transformed value for the experimentally calculated ES RNA concentrations obtained from these replicate runs. The clinically important cut-offs for the log difference between the standard input and calculated values are indicated by the dashed lines. The two outliers likely represent technical artifacts.

Additionally, different quantitative analysis was applied to the experiment described above. In particular, the VQA samples were entered into the LightCycler® software as unknown samples and quantified using the standard curve generated with ES RNA. Mean concentration values of the triplicate viral load measurements (i.e. each of five VQA samples were repeated three times within one RT-PCR run) were obtained for the VQA RNA copy controls. Table 2.3 shows the absolute and $\log_{10}$ transformed viral load values obtained for the VQA copy controls using the LUX and COBAS Amplicor assays (i.e. VQA assigned values). Absolute $\log_{10}$ differences obtained are indicated in the last column of the table. The VQA sample of $1.5x10^2$copies/ml was below the quantitation limit of the LUX assay.

2.3.3 Evaluation of the LUX assay using clinical specimens obtained from the patients in South Africa

2.3.3.1 Specificity

Specificity of the primers used for the LUX assay was estimated using the BLAST program (http:/www.ncbi.nlm.nih.gov/BLAST) that showed high degree of homology of the primer sequences to HIV-1 sequence only. Specificity of the LUX assay was evaluated using seronegative plasma samples obtained from 50 HIV-1 seronegative patients. All HIV-1 negative samples were tested in duplicate – with and without an IC. No HIV-1 amplification product was detected in any of the samples using HIV-1 specific primers – SKT145/150. IC was amplified in all the duplicate samples showing no PCR inhibition in these samples. Therefore, no false positive results were observed for this group of known HIV-1 negative patients using the LUX assay (Figure 2.13).

Table 2.3: HIV-1 viral load for VQA Copy Controls using LUX assay

VQA HIV-1 RNA Copy Controls (COBAS Amplicor viral load values in copies/ml)	VQA HIV-1 RNA Copy Controls (log_{10} values of COBAS Amplicor viral load)	VQA HIV-1 RNA Copy Controls (viral load obtained using LUX assay*, copies/ml)	VQA HIV-1 RNA Copy Controls (corresponding log_{10} values of LUX viral load)	Absolute log_{10} difference (log_{10} LUX assays minus log COBAS Amplicor)
$1.5x10^3$	3.2 log_{10}	$1.0x10^3$	3 log_{10}	- 0.2 log_{10}
$1.5x10^4$	4.2 log_{10}	$3.5x10^4$	4.5 log_{10}	0.3 log_{10}
$1.5x10^5$	5.2 log_{10}	$1.3x10^5$	5.1 log_{10}	- 0.1 log_{10}

*Mean of the triplicate viral load readings

2.3.3.2 Comparison of the LUX assay to the COBAS Amplicor assay using a random group of 142 HIV-1 positive patients

A random group of 142 HIV-1 positive patients was used to compare the performance of the LUX assay with the COBAS Amplicor assay. All samples obtained from the 142 patients were tested using a standard procedure for the COBAS Amplicor assay that provides a linear dynamic range of <400 - > 750 000 copies/ml. These samples were tested with the LUX assay in duplicate – with and without the addition of IC. IC was amplified in all samples tested thus showing no PCR inhibition in these samples. Samples from the VQA copy control panel were often included in the LUX assay runs to control for accuracy of quantitation (Figure 2.14).

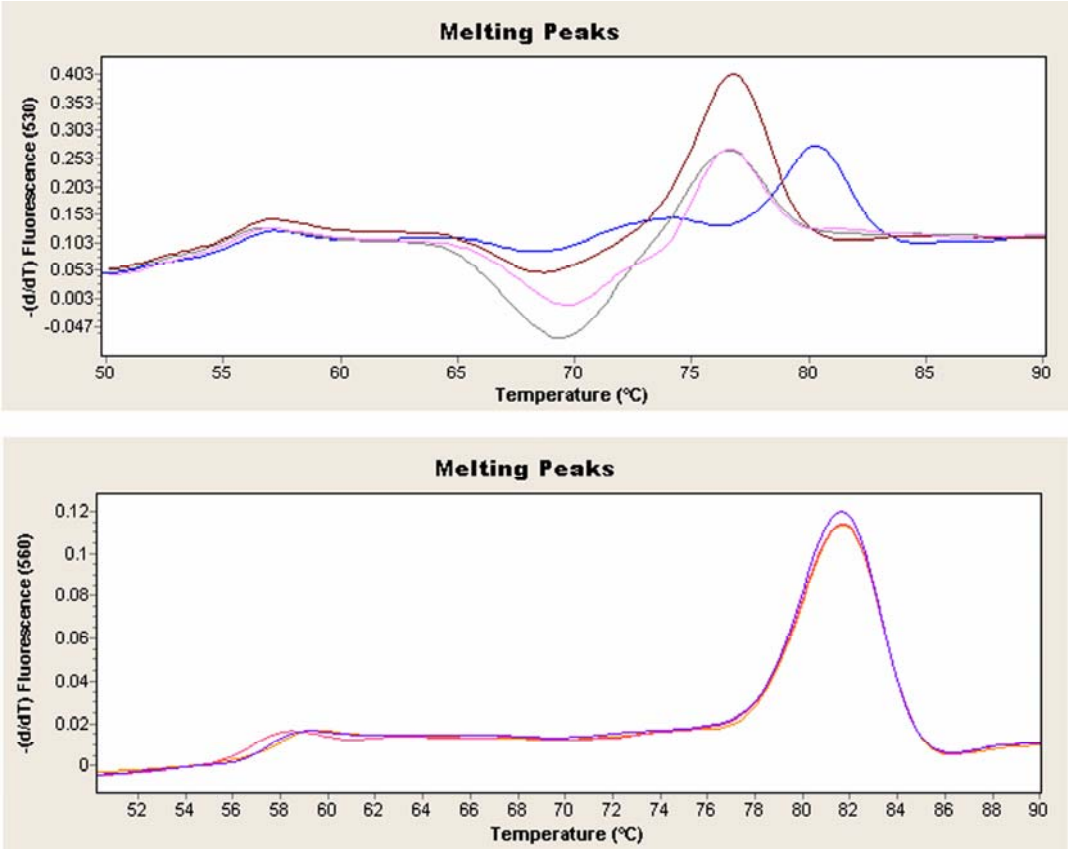


Figure 2.13: Melting curve analysis performed using HIV-1 seronegative samples.
Melting curve post-PCR profile is shown for three HIV-1 negative samples and an ES RNA standard. ES sample has a melting peak at ~80.5ºC (HIV specific product) when using 530 nm filter and the negative samples have a melting peak at ~76ºC (primer dimers). Melting curve analysis using filter 560 nm shows amplification product of IC (~ 82ºC) in the duplicate negative samples thus confirming the absence of RT-PCR inhibition.

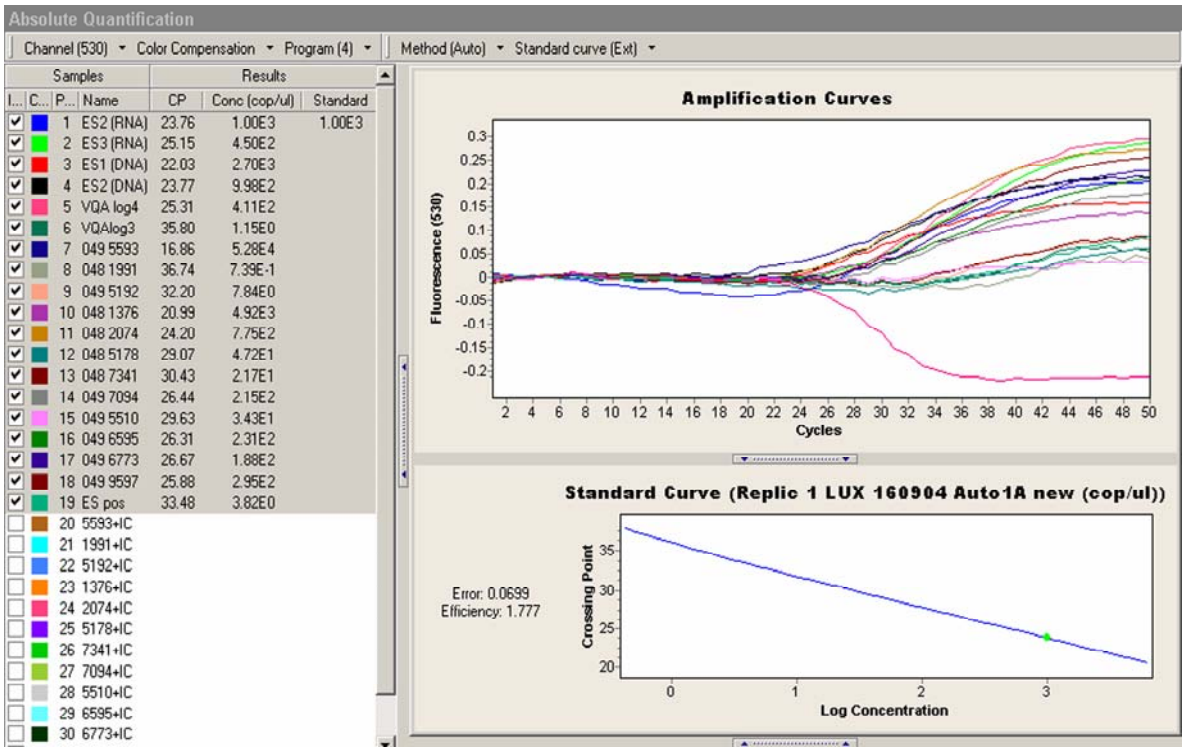
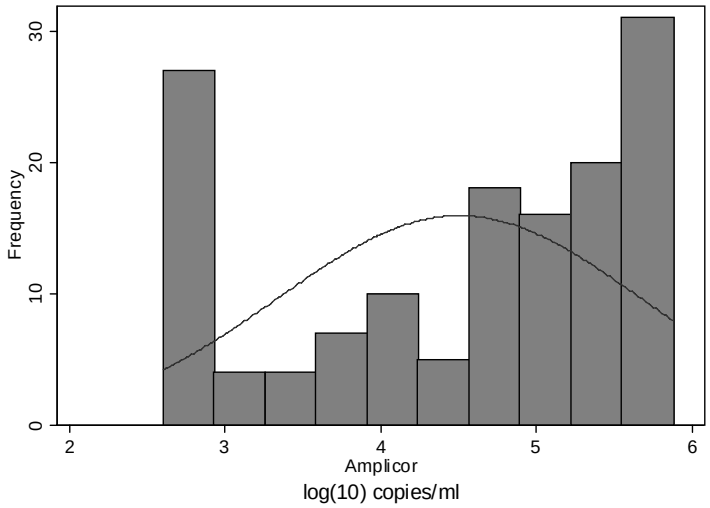


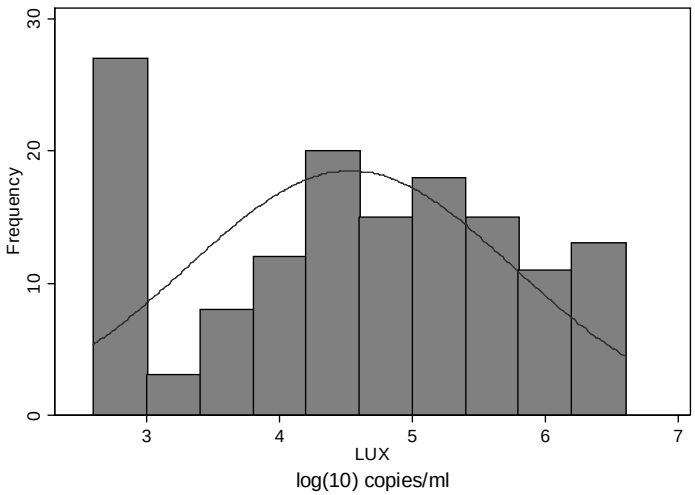
Figure 2.14: Captured image from the LightCycler® showing post-PCR quantitative analysis. Viral load values (copies/µl) are quantified by the LightCycler® software for the quantitation standards (ES; samples 1-4), the two VQA samples (samples 5 and 6) and patient's samples (7-18), sample 19 – ES RNA used as a positive control. Unselected samples represent duplicate patient tests containing IC (samples 20 30).

Statistical analysis of the $\log_{10}$ transformed viral load data was performed for both assays. The frequency graph (Fig. 2 A) for this group of patients (n = 142) tested with the COBAS Amplicor assay reflected a typical bimodal distribution of viral load values in the population receiving ARV therapy, with a mode (peak) at 2.6 $\log_{10}$ (the lower quantitation limit) and 5.8 $\log_{10}$ (the higher detection limit) values. The LUX assay distribution is trimodal (Fig. 2 B) with modes at 2.6 $\log_{10}$ and 4 – 5 $\log_{10}$ of viral load values. The COBAS Amplicor standard assay and the LUX assay have the same lower limit of detection (400 copies/ml; 2.6 $\log_{10}$) and hence similar grouping of values at the lower viral load range (2.6 $\log_{10}$) is demonstrated by both assays (Fig.2 A and B). Frequency distribution analysis (Fig. 2 A, B) shows some difference in the distribution of the results

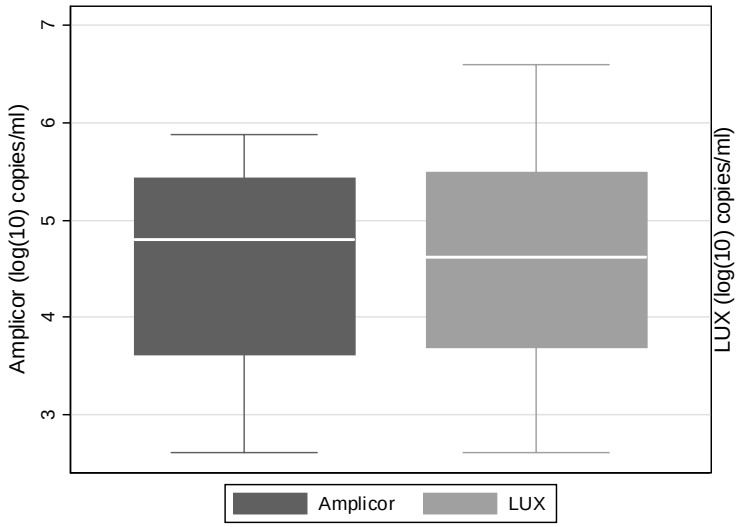
mostly at the higher viral loads obtained with both assays. In particular, the COBAS Amplicor has a modal (peak) at 5.8 log log_{10} and the LUX assay viral load values for these samples are more evenly distributed from 4 - 6.6 log_{10}. Figure 1C demonstrates similarity in the frequency distribution of HIV-1 viral load values obtained with both the COBAS Amplicor and the LUX assays (n = 142). The two assays have similar lower and upper quartile values though the COBAS Amplicor has a slightly higher median value then the LUX assay.



A



B



C

Figure 2.15: Histograms and box plots of the log10 viral load values for the COBAS Amplicor and the LUX assays. Graph A shows the frequency distribution (number of log_{10} viral load values on the vertical axis) of the log transformed HIV-1 viral load values (log_{10} copies/ml on the horizontal axis) obtained for the group of 142 patients using the COBAS Amplicor assay. Graph B shows the distribution of log_{10} values obtained with the LUX assay for the same group (n = 142). The combined graph (C) illustrates the distribution for both assays in the form of box plots. The distributions for both assays are quite similar (i.e. similar upper and lower quartiles). The LUX assay shows more symmetry and slightly more variability due to the higher viral load values of the upper quantitation limit ($4x10^6$copies/ml).

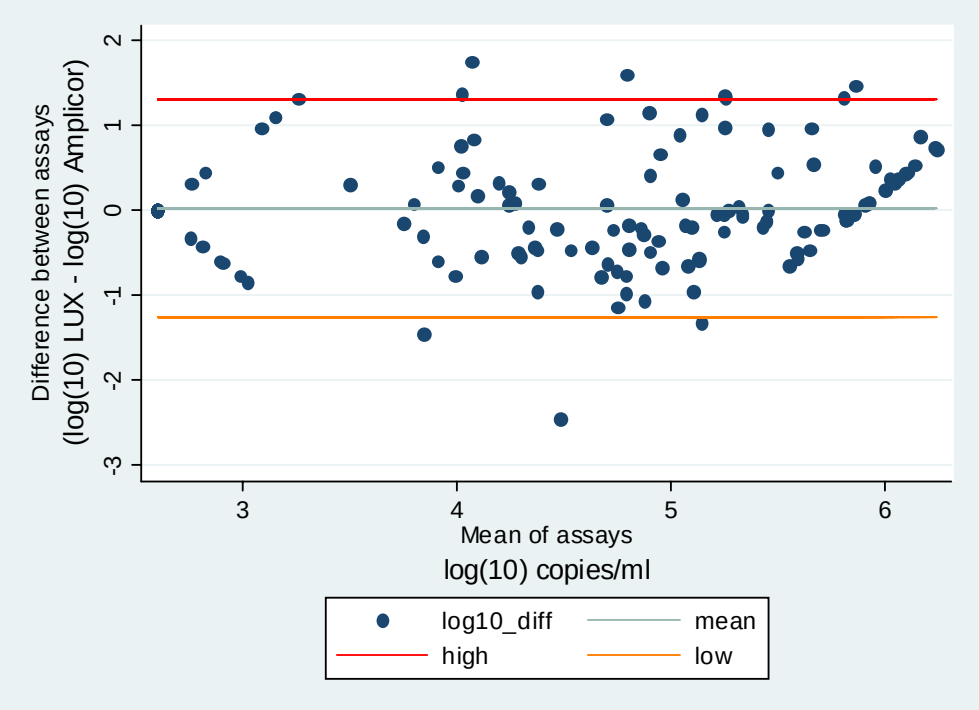


Figure 2.16: Bland-Altman model for measuring agreement between the LUX and COBAS Amplicor assays. The difference of $\log_{10}$ values between the LUX assay and the COBAS Amplicor assay (Y- axis) was plotted against the mean $\log_{10}$ value of the results obtained with both assays (X- axis). Mean difference between the log values is 0.03 and the limits of agreement for 95% of the data are from – 1.26 to 1.32 (CI 95%).

Agreement between the COBAS Amplicor and the LUX assays was established using the intra-class correlation (ICC) analysis. ICC coefficient of 0.85 with the confidence interval (CI) of 95% [80; 90] was obtained for this cohort. In addition, agreement between the assays was assessed using a Bland - Altman model (Bland and Altman, 1986) as displayed in Figure 3. The mean difference between the $\log_{10}$ viral load values of the LUX assay minus the COBAS Amplicor $\log_{10}$ values for 142 samples analyzed was 0.03 (limits of – 1.26 to 1.32). Based on the Bland – Altman analysis ~ 65% of the $\log_{10}$ paired differences were ≤ absolute 0.5 $\log_{10}$ (this includes both + 0.5 $\log_{10}$ and – 0.5 $\log_{10}$ intervals) and another ~ 22% of the values were within the interval of absolute ≥ 0.5; ≤ 1 $\log_{10}$ difference (this includes both plus and minus intervals). The remaining 13% of the values (18

samples) showed higher variability (≥ absolute 1 $\log_{10}$). Amongst these samples 5 were under-quantitated and another 13 samples were over-quantitated by the LUX assay when compared to the COBAS Amplicor assay.

2.3.3.3 Evaluation of the LUX assay using a cohort of 55 patients on HAART

A cohort of 55 patients on HAART, followed up longitudinally over five sequential visits (weeks 0, 4, 8, 12 and 24) was analyzed. The samples from the visit on week 24 were tested in duplicate using the LUX assay – with and without IC. No inhibition of PCR was found in all the duplicate samples. The samples for the baseline viral load measurement (visit on week 0) were tested using the standard version of the COBAS Amplicor assay with the linear dynamic range of 400 - 750 000 copies/ml. The follow-up samples (week 4, 8, 12 and 24) were tested using the ultra sensitive version of the Roche Amplicor assay, which has the linear dynamic range of 50 - 100 000 copies/ml. Once anti-retroviral (ARV) treatment had commenced the viral load values dropped significantly by week 4 in the vast majority of patients, from a median 5.44 $\log_{10}$ to 2.40 $\log_{10}$ and $\log_{10}$ 5.43 to $\log_{10}$ 2.60 for the COBAS Amplicor and the LUX assay, respectively (Fig.4). Five patients (n_1-n_5) out of 55 showed viral rebound at weeks 4, 8, 12 and 24, respectively. Table 1 shows that the increased viral loads detected in these patients by the COBAS Amplicor assay were also detected by the LUX assay. For patient n_1 (samples from the visit on week 4), the ultrasensitive COBAS Amplicor showed a viral load value of 5.0 $\log_{10}$, which corresponds to the upper quantitation limit of the test (100 000 copies/ml). Thus the corresponding LUX viral load reading of 4 000 000 copies/ml represents a plausible value. In patient n_2 the LUX assay showed consistently lower viral load readings for the baseline sample as well as for the sample on the week 12 visit reflecting a "spike" in viral load. In patients n_3 and n_5 the LUX assay when compared to the gold standard showed higher viral load values

for both baseline and weeks 12 and 24 respectively. For patient n_4 the LUX assay detected an increase in viral load for the week 24 visit, but the corresponding value obtained with the ultra sensitive COBAS Amplicor test was higher.

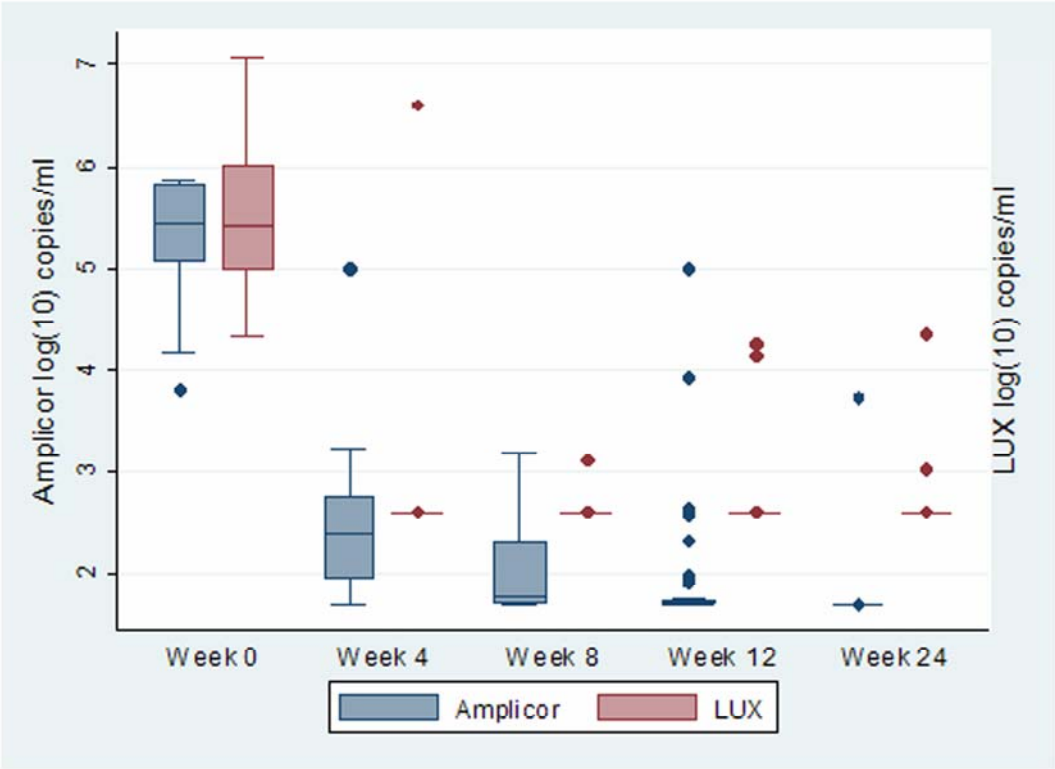


Figure 2.17: Box plots showing changes in viral load during longitudinal follow-up of 55 patients on HAART. At the baseline visit (week 0) both assays showed very similar median values of 5.44 $\log_{10}$ (COBAS Amplicor) and 5.43 $\log_{10}$ (LUX assay). From the next visit (week 4) onwards both methods show the decrease in viral load values to the lower quantitation limits – 2.40 $\log_{10}$ (COBAS Amplicor) and to 2.60 $\log_{10}$ (LUX assays). Individual patients revealed "spikes" ("blips") in viral loads detected on different visits.

Table 2.4: Follow-up samples from the patients showing viral rebound. Values indicating viral rebounds are in bold.

Patient	**Visit**	**Amplicor HIV-1 Monitor™ Viral load in log_{10} (copies/ml)**	**LUX assay Viral load in log_{10} (copies/ml)**
n_1	week 0	5.60 log_{10}	6.60 log_{10}
	week 4	**5.00 log_{10}**	**6.60 log_{10}**
	week 8	2.21 log_{10}	2.60 log_{10}
	week 12	1.70 log_{10}	2.60 log_{10}
	week 24	NA*	NA
n_2	week 0	5.79 log_{10}	4.65 log_{10}
	week 4	2.62 log_{10}	2.60 log_{10}
	week 8	1.77 log_{10}	2.60 log_{10}
	week 12	**5.00 log_{10}**	**4.14 log_{10}**
	week 24	NA	NA
n_3	week 0	5.88 log_{10}	6.37 log_{10}
	week 4	2.94 log_{10}	2.60 log_{10}
	week 8	3.20 log_{10}	2.60 log_{10}
	week 12	**3.92 log_{10}**	**4.26 log_{10}**
	week 24	NA	NA
n_4	week 0	5.88 log_{10}	6.38 log_{10}
	week 4	3.10 log_{10}	2.60 log_{10}
	week 8	2.64 log_{10}	2.60 log_{10}
	week 12	2.64 log_{10}	2.60 log_{10}
	week 24	**3.74 log_{10}**	**3.02 log_{10}**
n_5	week 0	4.98 log_{10}	5.34 log_{10}
	week 4	2.60 log_{10}	2.60 log_{10}
	week 8	1.83 log_{10}	2.60 log_{10}
	week 12	1.70 log_{10}	2.60 log_{10}
	week 24	**3.72 log_{10}**	**4.35 log_{10}**

NA* - not available

2.4 Discussion

This chapter describes development and evaluation of a new in-house assay for quantitation of HIV-1 RNA in plasma using the LightCycler® platform for real-time PCR. Modern real-time PCR technology allows flexibility of developing new, more affordable diagnostic assays that show good performance characteristics when compared to gold standard assays. A literature review revealed description of two real-time PCR systems used most frequently for the development of in- house assays for HIV-1 and HIV- 2 viral load quantitation – ABI Prism Sequence Detection System (models 7000, 5700 and 7700) from Applied Biosystems (Drosten et al., 2006; Ferns and Garson, 2006; Israel-Ballard et al., 2005; Palmer et al., 2003; Rouet et al., 2005; Schutten et al., 2000; Shapshak et al., 2005) and the LightCycler® real-time PCR system from Roche Applied Science (Demond et al., 2002; Gibellini et al., 2006; Gibellini et al., 2004; Gueudin et al., 2003; Gueudin and Simon, 2005; Ruelle et al., 2004). Among the two published LightCycler® methods for HIV-1 viral load quantitation, one was developed for different subtypes within group O (Gueudin et al., 2003; Gueudin and Simon, 2005) and another method for HIV-1 group M, subtype B (Gibellini et al., 2004). The assay developed by Gibellini et al (2004) uses a conserved region within the HIV-1 *gag* gene as an amplification target and the non-sequence specific SYBR Green detection format. However, both reported LightCycler® assays for HIV-1 viral load quantitation (Gibellini et al., 2004; Gueudin et al., 2003; Gueudin and Simon, 2005) did not use an internal control and were evaluated using limited number of clinical specimens.

Initial development of an in-house HIV-1 viral load assay was performed using an early version of the LightCycler® platform (version 1.2) and a FRET detection format (FRET assay). According to the assay design, quantitation of viral RNA copies is performed using

an external standard ES RNA for quantitation and control for RT-PCR inhibition is achieved by the addition of an IC to each reaction. Even though viral RNA extracted from one of the well characterized HIV-1 panels can be employed for quantitation, the use of a small synthetic RNA molecule as ES has certain advantages. Non- infectious i*n vitro* transcribed ES RNA can be produced at very high titers (e.g. 10^{11} copies/µl) for long term storage. Since the exact length of the synthetic ES RNA molecule is known, concentrations of a stock solution can be precisely established, which is crucial for quantitative RT-PCR. Furthermore, synthetic RNA can be chemically modified to dramatically improve its stability during storage even at lower temperatures (e.g 4˚C). Both ES and IC were designed and produced specifically for this viral load test. ES represents *in vitro* transcribed RNA and includes a conserved sequence from the p24 region of the HIV-1 *gag* gene. To ensure accurate and reliable quantitation using the in-house assay, ES RNA was extensively purified and quantified using a fluorescent dye for best sensitivity. Optimization of the FRET assay was performed using ES RNA and included RT-PCR amplification conditions, reaction volume, concentrations of primers and probes and stabilization of ES RNA. Although version 1.2 of the LightCycler® instrument recommends performing amplification reactions in 20 µl volume a dramatic improvement in sensitivity of the FRET assay was demonstrated using 30 µl reactions. Next, titrations of a competitive IC showed that only 3 logs of ES RNA concentrations can be co-amplified and reliably quantified in the presence of either *in vitro* RNA IC or a DNA equivalent of IC. In an attempt to improve the dynamic range of an internally controlled FRET assay, the competitive IC was modified into a non-competitive IC by replacing the primer binding sites in the former. Titrations of a non- competitive IC DNA revealed the most optimal concentration of IC primers and IC DNA that can co-amplify with 5 logs of ES RNA concentrations (i.e. $1.5x10^3$ to $1.5x10^7$ copies/ml). A DNA IC was evaluated instead of an

RNA IC due to its higher stability at lower concentrations and lower costs involved in maintaining the stocks. DNA IC similarly to RNA IC would reveal inhibition of PCR. However, this approach lacks the advantage of controlling for inhibition during Reverse Transcription.

Preliminary evaluation of the FRET assay was performed using ES RNA, VQA copy controls and the BBI panel. Preliminary evaluation of specificity of the FRET assay was based on a BLAST search, which showed that the sequences of primers and probes used for the assay are highly homologous to HIV-1 *gag* sequence only. Quantitative performance of the FRET assay was evaluated in five experiments repeated on different days using serially diluted ES RNA. Five RNA standards containing non-competitive IC and covering quantitation range from $1.5x10^3$ to $1.5x10^7$ copies/ml were each repeated six times within each RT-PCR run. Based on these repeated experiments the sensitivity, linearity of the dynamic range and reproducibility of the FRET assay was investigated. Quantitation limit of the FRET assay in the presence of IC is $1.5x10^4$copies of ES RNA per ml, which indicates rather insufficient sensitivity of the assay. The dynamic range of the assay is linear ($R^2 = 0.98$; Figure 2.9) over four orders of magnitude from $1.5x10^4$ to $1.5x10^7$ copies/ml. The average inter-run variability was 21%, indicating that the assay is reproducible. Further evaluation of the FRET assay was performed using the VQA HIV-1 RNA Copy Controls consisting of subtype B virus. No real-time detection and quantitation was observed for all five VQA samples of different concentrations. However, the RT-PCR products from VQA samples of $1.5x10^4$ and $1.5x10^5$copies/ml could be visualized on the agarose gel, confirming successful amplification. This phenomenon may be explained by the presence of sequence mismatches and thus insufficient hybridization of the FRET probes to the amplicons generated with primers SKT145/150. FRET hybridization probes

usually cover up to 60 bp, and in this particular application probe design was problematic due to the high sequence diversity amongst HIV-1 group M isolates. Since HIV-1 subtype C is the predominant subtype circulating in South Africa (Papathanasopoulos et al., 2003) the FRET probes were designed based on known subtype C polymorphisms within the target *gag* region. In order to accommodate for such polymorphisms and make the detection of this subtype more optimal degenerate nucleotides were included in the sequence of FRET probes. Next, validation of an in-house quantitation standard (ES RNA) was performed using the BBI panel represented by subtype C HIV-1. Minor differences in viral load values were obtained for the BBI panel when tested with the FRET assay in triplicate using a real-time and an imported standard curve. An average viral load value for the panel was $\log_{10}$ transformed and compared to a commercial RT-PCR method – COBAS Amplicor assay. An absolute difference between the two methods was 0.3 $\log_{10}$ indicating that quantitation of HIV-1 RNA provided by the FRET assay compares well to the gold standard assay. Overall, the FRET assay showed good reproducibility and good accuracy of quantitation using the BBI panel. The assay is also internally controlled to prevent false positive results. However, insufficient sensitivity and specificity of detection of the FRET assay restricted to subtype C required an improvement.

Further development of the LightCycler® viral load assay was performed using a new, more advanced version (version 2) of the platform. The latest capillary based version allows the use of a wide variety of real-time detection formats as well as larger reaction volumes (e.g. 20 – 100 µl). Therefore, transfer of the originally developed FRET assay onto the new version of the instrument was conducted with a view of improving sensitivity and specificity of the assay. In order to avoid the effects of possible polymorphisms in the HIV-1 *gag* sequence, the FRET detection format used initially for the in-house viral load

assay was replaced with a LUX™ primer format (LUX assay). The use of a LUX™ primer format for this purpose is novel and holds a number of advantages. The fluorescently labeled self-quenched LUX™ primer does not require a separate quencher (i.e. it is mono-labeled) and combines the features of a primer and a probe (Chen et al., 2004; Donia et al., 2005; Lowe et al., 2003; Nazarenko et al., 2002b; Nazarenko et al., 2002a; Sharkey et al., 2004), which makes it a more cost effective option when compared to other real-time detection formats. The LUX™ primer is also more suitable for detection of sequences in which polymorphisms are present in abundance, for example HIV-1 sequence. In our application, HIV-1 specific primers SKT 145/150 are located within a conserved region of the *gag* gene and thus conversion of one of these primers into a LUX™ format can provide better specificity of detection within subtype C as well as across different subtypes of virus. As opposed to SYBR Green dyes, TaqMan probes and Molecular Beacons, the LUX™ primer offers a combined possibility for multiplexing and performing a melting curve analysis (Sharkey et al., 2004). Similarly to SYBR Green dyes a LUX™ primer has reduced specificity due to detection of PCR byproducts like primer dimers. Formation of primer dimers is often inevitable in quantitative PCR that has a wide quantitation range, especially at low concentrations of template. This issue of detecting non-specific PCR products with a LUX primer was addressed in previously published studies (Lowe et al., 2003; Nazarenko et al., 2002b). During optimization of the LUX assay this problem was solved by including a fourth segment at the end of each PCR cycle. This additional denaturation segment (78˚C for 4 seconds) allowed acquisition of a fluorescent signal at a temperature that exceeds the melting point of primer dimers, but is lower than the melting point of amplicons. This modification to the real-time RT-PCR profile ensured accurate and specific detection and quantitation of HIV-1 amplicons using the LUX SKT 150 primer. Optimization of the LUX assay also revealed that addition of IC DNA or RNA

even at a low copy number reduces the dynamic range of the assay and affects accuracy of quantitation at the lower ES RNA concentration range (data not shown). Thus, in order to maintain good sensitivity the modified design of the LUX assay includes the use of a DNA IC in a duplicate test sample, which is not used for HIV-1 RNA quantitation. A duplicate test is used only to control for false negative results. Preliminary evaluation of the LUX assay using serial dilutions of ES RNA revealed a LLQ of 400 copies/ml. Significantly improved sensitivity of the LUX assay when compared to FRET assay (400 copies/ml versus 15 000 copies/ml, respectively) for the most part due to the larger reaction volume allowing greater input of RNA template. The sensitivity level of the LUX assay is comparable to the West African in-house viral load assay that has a quantitation limit of 300 copies/ml and makes use of the ABI Prism technology (Rouet, et al., 2005). The LightCycler in-house viral load assay developed by Gibellini and colleagues provides a sensitivity of 50 copies/ml. using an older version of the platform, and 20 µl of a total reaction volume. High sensitivity of this assay may be attributed to the larger volumes of patient plasma (1 ml) used for HIV-1 RNA extraction (Gibellini, et al., 2004). In general, sensitivity and reproducibility of RT-PCR at low copy numbers is affected by a number of factors. Low copy number of the initial template molecules is influenced by statistical distribution effects, as described by Poisson's distribution law, and therefore only some reactions will have the correct input copy number (Morrison et al., 1998; Saha et al., 2001). For example, a concentration of 1.5 x 10^3copies/ml corresponds to ~ 45 input copies in a 30 µl volume. This small number becomes more variable due to stochastic effects when pipetting small template volumes. Moreover, variable RT efficiency reduces the number of initial targets for cDNA synthesis and further limits the sensitivity of RT-PCR (Hayward et al., 1998). Repeated experiments performed for the LUX assay using ES RNA dilutions from 4x10^2 to 4x10^6copies/ml showed good reproducibility of the assay (Table 2.2).

Reproducibility of the LUX assay was also analyzed using $\log_{10}$ transformed data (Figure 2.12) and showed that overall variability (intra- and inter-assay variability) of the assay, except for a few outliers did not exceed 0.5 $\log_{10}$ copies. For quantitative assays, a 3-fold variation (0.5 $\log_{10}$ copies) is accounted for by intra-assay variability and biological variability, but clinically a 10-fold (1-$\log_{10}$) difference is regarded as clinically significant (Martin, 2000). The significance of 1-$\log_{10}$ variability in the assay is that it may lead to the clinical mismanagement of a patient on ARV treatment. Although the LUX assay shows an increased variability at lower quantitation levels and some variability between the 5.6 $\log_{10}$ and 6.6 $\log_{10}$ range this is within acceptable ranges (< 0.5 $\log_{10}$). Thus, no clinically significant variability was found for the LUX assay. Preliminary evaluation of the quantitative performance of the LUX assay and an additional validation of an in-house quantitation standard (i.e. ES RNA) was conducted using the HIV-1 RNA Copy Controls from the VQA laboratory. As opposed to the FRET assay that failed to detect and quantitate the VQA samples, the LUX assay was successful in both real-time detection and quantitation. Similarly to the BBI panel tested with the FRET assay, the VQA samples showed that amplification efficiency of ES RNA is comparable to that of HIV-1 RNA, which is essential for quantitative PCR. Testing of the VQA copy control samples also demonstrated that the LUX assay quantified HIV-1 viral load in these samples in the same $\log_{10}$ range as the Ultra sensitive Roche Amplicor HIV-1 Monitor™ v1.5 test and with a maximum absolute $\log_{10}$ difference of 0.3 $\log_{10}$ (Table 2.3). Thus, preliminary evaluation showed that the LUX assay compares well to the in-country gold standard test.

Preliminary clinical evaluation of the LUX assay was performed using a total number of 458 clinical specimens collected from the South African population. The DNA IC was run in duplicate tests for 247 out of the 458 samples (due to costs), and confirmed there was no

PCR inhibition, while maintaining the good quantitative features of the LUX assay. However, future development and implementation of the LUX assay must aim to maintain good quantitative characteristics by spiking every sample with an RNA IC prior to extraction in order to provide full control for false negative results.

To establish whether the assay would be applicable in individuals infected with non-C subtypes, a comparison of the primer sequences with all available sequences of HIV in the Los Alamos database (http://www.hiv.lanl.gov) was conducted. The primers bind in highly conserved regions of p24 of all isolates, with the exception of subtype G (single base mismatch at the 3' end of the SKT150 primer). Since only a few subtype G, and G recombinants have been identified in South Africa (Bredell et al., 2002; Papathanasopoulos et al., 2002), the LUX assay is expected to work for the majority of HIV-1 infected individuals. Future evaluations of this assay will include degenerate SKT150 primer designs to ensure coverage of HIV-1 group M subtypes. The p24 region of HIV-1 *gag* has been well characterized as an amplification target in subtype C using the Roche Amplicor assay, versions 1.0 and 1.5 (Alaeus et al.,1999; Mechael et al., 1999; Parekh et al., 1999) and at the time of development of the in-house assay represented the best choice. However, recent literature suggests other conserved regions for amplification, e.g. LTR region of HIV-1 genome (Drosten et al., 2006). An ongoing molecular surveillance is necessary to identify sequences that are highly conserved in most non - B subtypes and in circulating recombinant forms (CRF). Determination of such a target in future studies may require some modifications to the current assay, which will ensure reliable quantitation of this highly divergent virus.

Good specificity of the LUX assay was shown using 50 HIV seronegative plasma specimens. Good quantitative performance of the assay was demonstrated using a random group of patients and a longitudinally followed-up cohort of patients receiving therapy. The analysis of a random group of patients (n = 142) revealed that both the described assay and the gold standard assay reflect overall similar distributions of viral load values (Figure.2 C). The differences in distribution at the highest values between the assays are largely due to an upper quantitation limit of the LUX assay being at 6.6 $\log_{10}$ (upper quantitation limit at 4 000 000 copies/ml) versus 5.8 $\log_{10}$ (upper quantitation limit at 750 000 copies/ml) for the COBAS Amplicor test. The LUX assay shows higher distribution (frequencies) in 4 $\log_{10}$ (middle range of the LUX assay) when compared to the gold standard. This could be attributed to the different types of quantitation involved – real-time when using the LUX assay versus an end point quantitation when using the COBAS Amplicor. The agreement between the LUX assay and the COBAS Amplicor assay was studied using an intra class correlation (ICC) coefficient and the Bland – Altman method (Figure. 3). Good agreement appears to exist between the two assays since a high value for the ICC was found (0.85, 95% CI: 0.80 – 0.9). The Bland – Altman model revealed a mean bias of 0.03, which reflects good accuracy of quantitation using the LUX assay. In total, approximately 87% of the values showed less than absolute 1 $\log_{10}$ difference, which is not considered a clinically significant difference (Martin, 2000). Amongst values that showed higher variability (> absolute 1 $\log_{10}$), ~3.52% (5/142) were under-quantitated and ~9.15% (13/142) were over-quantitated by the LUX assay. Due to the lack of availability of retrospective samples it was not possible to perform DNA sequencing and thus exclude nucleotide mismatches in primers and/or probe binding sites as a likely reason for under quantitation observed in both assays. Some degree of viral RNA degradation in the stored, retrospective samples may contribute to the under- quantitation observed with the LUX

assay. Therefore, the Bland-Altman analysis shows good agreement between the LUX assay and the COBAS Amplicor assay for ~ 87% of the results.

Clinical usefulness of the LUX assay in monitoring HIV-1 viral load was evaluated using the cohort of 55 patients on ARV treatment followed-up longitudinally. For this cohort of samples an automated MagNA Pure extraction system was used. The system utilizes magnetic glass particles for capture and detection of nucleic acid molecules and provides good efficiency using small volumes of plasma (Espy et al., 2001; Mohammadi et al., 2003). For the baseline samples that were collected prior to treatment initiation both the standard COBAS Amplicor and the LUX assay showed median viral load values of 5.44 $\log_{10}$ and 5.43 $\log_{10}$, respectively. A dramatic decrease in viral load values was observed in most patients 4 weeks after initiation of treatment indicating good response to the combination therapy used. This was reflected similarly by an ultra sensitive version of the COBAS Amplicor and the LUX assay that showed viral load values of 2.40 $\log_{10}$ and 2.60 $\log_{10}$, respectively. HIV-1 viral load values below the detection limit of both assays were demonstrated in these patients during the subsequent visits (weeks 8, 12 and 24). During the course of therapy, 5 patients out of 55 showed an increase in HIV-1 viral load values. Both the ultra sensitive COBAS Amplicor and the LUX assay revealed viral rebound in these patients using the samples obtained on the same visit (Table 2.4). Even though the LUX assay in combination with the MagNA Pure extraction system uses 3.5 times less plasma than the ultra sensitive COBAS Amplicor assay, it provides sufficient sensitivity for early detection of viral rebound in patient's on ARV treatment. Therefore, in the patients on HAART the LUX assay shows a similar downward trend in viral load values over time as the COBAS Amplicor assay (Figure 2.17). Most importantly, the "spikes" in HIV-1 viral load that occurred in five patients were all detected similarly by both the ultra

sensitive COBAS Amplicor assay and the LUX assay. This evaluation demonstrated good clinical performance of the LUX assay in monitoring the efficacy of ARV treatment in the patients during longitudinal follow-up.

In conclusion, this study suggests that the LUX assay represents a more affordable alternative tool for monitoring HIV-1 viral load in South African patients on antiretroviral therapy. Preliminary cost estimation of the developed assay includes: cost for RNA extraction ~$5.7, RT-PCR reagents ~ $5.7, consumables ~ $ 3.4, cost for labor ~ $8.9. The total cost is ~ $ 23.7 per test, which is approximately 2 to 3 times less expensive than the commercial assays currently used in the public sector in South Africa (~$ 45 for EasyQ, Biomerieux; $ 75-80 COBAS Amplicor, Roche). Future development of the LUX assay will aim to further reduce the cost of the assay. It is also envisaged to transfer the assay onto the latest version of the LightCycler® system – version 480 and evaluate the LUX assay using fresh plasma samples. The LightCycler® 480 offers a significantly higher throughput (96 or 384 samples per run) than the approved commercial assay (Table A1, Appendix A). An ultimate goal of this study is to provide the high volume laboratories in South Africa with an affordable, high throughput assay with performance characteristics comparable to those of the commercial assays. The development and preliminary clinical evaluation of the LUX assay is the first step towards achieving this goal.

CHAPTER 3

Evaluation of two commercially available, inexpensive alternative assays used for quantitation of human immunodeficiency virus type 1 (HIV-1) in plasma

This chapter describes the evaluation of the heat-denatured (HD), signal-boosted p24 antigen assay (the HiSens™ HIV-1 p24 Ag Ultra assay) and the reverse transcriptase (RT) enzyme activity assay (the ExaVir™ Load assay) using a total number of 117 plasma specimens. These specimens were obtained from a cohort of 20 HIV-1 positive patients (89 samples) on HAART followed up longitudinally, and 28 treatment-naïve patients from South Africa. Performance of both alternative viral load assays was compared to the Roche Amplicor HIV-1 Monitor™ (version 1.5) assay. The main objectives of this component of the study were to:

- Evaluate the performance of these surrogate viral load assays in HIV-1 subtype C, which is the predominant subtype in South Africa;
- Evaluate the usefulness of these assays for monitoring HIV-1 viral load in patients on HAART;
- Assess the technical complexity and ease of implementation of these assays in medium tier district laboratories in South Africa.

3.1 Introduction

The South African National Antiretroviral Treatment Guidelines (National Department of Health, SA, 2004) recommend routine monitoring of HIV-1 viral load in patients on HAART. Approximately 10.3% of NHLS laboratories are classified as medium tier laboratories and do not have either the facilities or trained personnel for implementation of PCR or other NAT based viral load methods (see section 1.1). Thus, non-NAT based and technically less complex viral load assays represent a reasonable alternative for this group of laboratories and others in resource limited environments. Among such methods are the HiSens™ HIV-1 p24 Ag Ultra assay (Perkin Elmer) and the ExaVir™ Load assay (Cavidi).

One of the commonly used alternative viral markers, the p24 protein, is the chief component of the nucleocapsid core proteins translated from the same RNA species as is enclosed in virus particles (Hammer, 1996). The test principle for p24 antigen detection and quantitation is based on formation of the antibody-antigen-antibody sandwich, which is detected in an ELISA plate format. In particular, p24 antigen present in plasma or serum is added to a microtiter plate coated with p24-specific, usually monoclonal, capture antibodies. After incubation, unbound reaction components are washed away and bound antigen is reacted with another p24-specific "tracer antibody". The "tracer" antibodies are conjugated to an enzyme – horse radish peroxidase or alkaline phosphatase, which are capable of generating a colour signal when combined with a suitable substrate. The signal is detected as a change in the optical density of the reaction using a conventional ELISA plate reader (Schüpbach, 1999). The original version of the p24 quantitation assay was criticized for poor sensitivity and problems with generating false negative and false positive results (Lange et al., 1987). Insensitivity and/or false negative results of the p24

test were largely due to the immune complexes formed between of p24 antigen and anti-p24 antibodies in the serum of the infected individuals. Another problem was caused by the immunoglobulin-specific (anti-Ig), rheumatoid-factor-like antibodies which may bridge the capture and the tracer antibody in the p24 test thus generating overdetection or false positive results. Professor Schüpbach and his colleagues managed to overcome these problems by including a heat denaturation step in the p24 test. During this step the diluted sample is boiled at 100ºC for 5 minutes. Thus interfering antibodies (i.e. anti-p24 anti-Ig) are eliminated by heat-mediated destruction of their 3 dimensional structure (Schüpbach and Bone, 1993). The heat denaturation step and the use of plasma instead of serum increased the sensitivity of the p24 test. Further improvement was achieved by boosting the signal using tyramide-based signal amplification with the NEN ELAST® reagents. This ELISA reaction allows p24 quantification across a range from 0.5 pg/ml to more than 6 ng/ml (Schüpbach et al., 1996; Schüpbach et al., 2000). Further evaluation of the HD p24 assay for diagnosis of pediatric HIV-1 infection demonstrated that the assay was as sensitive and specific as nucleic acid based HIV-1 diagnostic assays (Lyamuya et al., 1996; Schüpbach et al., 1994; Sherman et al., 2004; Sutthent et al., 2003). Recent work has suggested that a modified p24 antigen assay can also be used on dried whole-blood spots allowing accurate, reliable, inexpensive and early diagnosis of HIV-1 in infants (Patton et al., 2006). Good performance of the HD p24 assay for longitudinal monitoring of the efficacy of ARV therapy has been demonstrated using a cohort of 23 patients on HAART where p24 antigen was detected as sensitively as viral RNA. On average the changes in the p24 antigen concentrations and viral RNA copies/ml correlated excellently (Boni et al., 1997). In a study reported by Ledergerber and colleagues, HD p24 quantitation results inversely correlated to CD4 counts and compared well to the concentrations of viral RNA showing that p24 is a marker of disease progression in adults (Ledergerber et al., 2000).

Moreover, the HD p24 test has been shown to have good specificity across different group M subtypes of HIV-1, including group O which is usually not detected by commercial RNA based assays (Burgisser et al., 2000). In three Swiss studies the improved HD signal-boosted p24 assay showed good association with RNA levels in treated adults and children (Schüpbach, 2002).

Another alternative viral load assay - ExaVir™ Load assay (Cavidi) uses a different marker of viral replication – HIV-1 reverse transcriptase (RT) enzyme activity. The ExaVir™ Load assay (RT assay) measures the activity of viral RT enzyme based on the quantity of product (DNA-RNA hybrid) synthesized by this enzyme. The earlier versions of this assay were hampered by the susceptibility of the RT enzyme to inhibitory antibodies and other interfering molecules (Burgisser et al., 2000). This problem was solved in the newer versions of the RT assay in which viral particles are first captured by a special gel and the remaining plasma containing the interfering substances is washed away. The assay procedure is divided into two parts: the plasma separation and the RT-assay. In the separation part of the assay plasma is treated to inactivate cellular enzymes and then incubated with a special liquid gel that binds the virion lipid membrane. Once viral particles are captured by the gel and the plasma containing disturbing products such as antibodies or anti-retroviral drugs is washed away. Captured virions are then lysed and the lysate containing RT enzyme is collected and used in the RT-assay step. The RT-assay is performed in 96-well plates coated with poly-A RNA molecules. The reaction mixture added to the plate contains primer, BrdUTP(s) (Bromo-deoxyuridine triphosphate) and viral lysates. In the presence of these entire reaction components active RT enzyme will synthesize DNA using an RNA template. The resulting DNA-RNA hybrid is detected in an ELISA set-up by the addition of anti-BrdU antibodies conjugated to alkaline phosphatase

(AP) and a colorimetric or fluorimetric AP substrate (Award et al., 1997; Braun et al., 2003; Garcia et al., 1998). The RT activities found in plasma samples are expressed relative to a reference RT in femtograms per millilitre plasma (fg/ml). Studies conducted using the first generation of the ExaVir™ Load kit and a Stockholm cohort of patients, mostly subtype B, showed a strong correlation between RT activity and RNA genome copies present according to the Roche Amplicor™ assay (Malstein et al., 2002). The specificity of the viral load assays measuring viral genome or protein levels is dependent largely on the sequence of primers, probes and the antibodies used. The Cavidi ExaVir™ Load kit circumvents this problem by measuring the activity of the RT enzyme, which is essential for virus replication and well conserved across most subtypes (Award et al., 1997; Corrigan et al., 1998). Evaluation of the most recent, improved version of the ExaVir™ Load kit (version 2) showed sufficient sensitivity of the RT assay for longitudinal follow up of patients on ARV therapy. Good association of the RT assay with current amplification assays – the Roche Amplicor and the Bayer bDNA assays was demonstrated. Moreover, this study revealed no significant effect of efavirenz therapy on RT assay performance despite efavirenz binding to the RT enzyme (Greengrass et al., 2005).

Evaluation of the ExaVir™ Load kit (version 2) as a tool for donor blood screening revealed 100% specificity of this assay. The fluorimetric version of the kit showed slightly better sensitivity than the colorimetric version. This study suggests that the detection of RT activity has a potential for use in blood screening in addition to other markers (Seyoum et al., 2005).

The alternative methods described above use different surrogate markers for quantitation of HIV-1 viral load, therefore, extensive evaluation of these alternative assays is required

prior to implementation in routine diagnostics. Technical aspects of these alternatives should also be considered.

3.2 Materials and Methods

3.2.1 Sample population (Total n = 117)

A total of 117 samples were collected from HIV positive individuals followed up at the Johannesburg hospital outpatient HIV clinic. All patients were from Johannesburg and surrounding areas and were all receiving HAART. Studies were conducted in full conformance with the local ethics committee approval (Appendix D). Clinical specimens were fresh and/or frozen plasma collected in vacutainer EDTA tubes. HIV Viral RNA was determined using the Amplicor HIV-1 Monitor™ assay version 1.5 (Roche Molecular Systems Inc., Branchburg, New Jersey, USA) as per manufacturer's instructions. In patients who presented with a viral load values below the lower quantitation limit of the assay (<400 RNA copies/ml), the ultra-sensitive version of the assay was performed which has a detection limit of 50 RNA copies/ml. In addition, residual samples (n = 117) stored at –70 º C were evaluated using both the heat denatured p24 ultrasensitive assay as well as the ExaVir ^{TM} Load assay as per manufacturer's instructions.

Eighty-nine of the available plasma samples, representing 20 patients, were selected due to the availability of serial samples while patients were being monitored on HAART. The majority of patients returned for at least 4 sequential visits with a number of patients continuing onto the 6th visit. Blood samples for viral load determinations were scheduled at baseline and weeks 4, 8, 12, 16, 24 and then 8 weekly thereafter. The number of samples analyzed at each of the six visits was 20; 20; 20; 15; 9 and 5 respectively.

3.2.2 Heat-dissociated, boosted p24 antigen assay

Measurements of the p24 antigen in plasma were performed using the HiSens HIV-1 p24 Ag Ultra kit **(**Perkin Elmer Life and Analytical Sciences, Turku Finland) as per manufacturer's instructions with some modifications. Briefly, 50 µl plasma was initially lyzed in 25 µl lysis buffer prior to being diluted 1:6 in 0.5% Triton X-100 (Schüpbach et al., 2003; Schüpbach et al., 2006). The lysis buffer (virus disruption buffer) used to conduct the assay was a new improved version kindly donated by Jorg Schüpbach (personal communication). The diluted sample was heat denatured for 5 minutes at 100ºC. Treated plasma samples (250 µl) were then transferred to wells of a microtiter plate coated with monoclonal antibody to HIV-1 p24 and incubated for two hours at room temperature on a micotiter plate shaker. Wells were washed with 1 x wash buffer, to which 100 µl of biotinylated detector antibody was then added and incubated for 1 hour at 37ºC. After washing, 100 µl of diluted S-HRP was added to all wells and incubated at 37ºC for 15 minutes. Following washing 100 µl of biotinyl tyramide working solution was added to each well and incubated for 15 minutes at room temperature to allow for signal amplification. Next, the diluted S-HRP was added and incubated for a further 15 minutes at room temperature. Finally, o-phenylenedianine (OPD) substrate was added to all wells after washing and the plate was inserted into the kinetic ELISA plate reader (Bio-Tek Elx800 microtiter) Quanti-Kin Detection System Software, (DL3, Diagnostica Ligure, Genoa, Italy) was used to perform kinetic readings over a 30-minute period. A further incubation at room temperature for 10 minutes in the reader was stopped by the addition of 100 µl stop solution and an end-point reading taken. The run was considered valid when the substrate blank had an OD $\leq$ 0.05, the negative control OD $\leq$ 0.10 and the 6103 fg/ml standard was OD > 0.15. The Quanti-Kin system incorporates programs for data reduction

and the results were obtained as printouts of calibration curves and concentrations for unknown samples.

3.2.3 ExaVir ™ Load Quantitative HIV-RT Load Kit

HIV RT-activity was quantified using the ExaVir™ Load kit (Cavidi Tech AB, Uppsala, Sweden) according to manufacturer's instructions. In the separation part 1ml of plasma was treated to inactivate interfering cellular enzymes. The viral particles were then captured and immobilized on a separation gel column and washed to remove other potential interfering factors. Isolated virions were lyzed with kit lysis buffer to recover the intraviral RT enzyme.

During the RT-assay a reaction mix containing oligodT prime, BrdUTP and the lysates were added to the 96-well Poly A-coated plate. Two different amounts of lysates, 75µl and 15µl were used in order to increase the detection range of the assay. Recombinant HIV-1 RT supplied with the kit was used as a standard for quantitation. A serially diluted standard of a known concentration was also added to the plate in 75µl and 15µl volumes. In the presence of RT, DNA-RNA hybrids could be synthesized by RT during an overnight incubation at room temperature. After washing, this product was detected with α- BrdU antibodies conjugated with an AP. The product was quantified by addition of a colorimetric AP substrate. Colorimetric readings were taken on a Bio-Tek Elx800 microtiter ELISA plate reader. The first reading was done immediately after the addition of the AP substrate to establish the baseline signal and then repeated two hours later. A final reading was taken after an overnight incubation. Viral RT activity was expressed as fg/ml.

These values were then converted to copies/ml equivalents by the software provided (Ledergerber et al., 2000).

3.2.4 Plasma HIV RNA Assay

The Amplicor HIV-1 Monitor^{TM} version 1.5 assay (Roche Molecular Systems Inc., Branchburg, New Jersey, USA) was used as the gold standard assay. For the standard and the ultrasensitive version of the assay 200 µl and 500 µl of plasma were used, respectively. The assay was performed according to manufacturer's instructions.

3.2.5 Statistical analysis

All statistical analyses were performed using $\log_{10}$ transformed values for all the variables in the analyses, i.e. $\log_{10}$p24, $\log_{10}$RT ($\log_{10}$fg/ml) and $\log_{10}$RNA ($\log_{10}$ copies/ml, Amplicor) except for CD4 counts. Schematic (box) plots of $\log_{10}$p24, $\log_{10}$RT and $\log_{10}$RNA over visits were constructed to evaluate the change in these values over time for all patients on treatment. Tests for trend of these values over visits were conducted using a mixed effects (repeated measures) model (Diggle et al., 1994) with orthogonal polynomial contrasts (Snedecor and W.G., 1989) for visits included in the model.

To assess the association between $\log_{10}$p24 and $\log_{10}$RNA, $\log_{10}$RT and $\log_{10}$RNA, we used a mixed effects (repeated measures) model for each association with a heterogeneous first-order autoregressive-correlation structure for the values within a patient. This allows for lower correlations between values that are further apart than those that are adjacent within a patient, as well as adjusting for the heterogeneity of the outcome values within a

patient. The coefficient of determination (R^2) as the square of the correlation between the observed and predicted values was calculated for each model. To show negative correlation between CD4 counts and all 3 viral load assays the Spearman test was applied. All statistical tests were two-sided and all statistical analyses were performed using the SAS V8.2 software.

3.3 Results

Four negative human plasma (NHP) samples of different lots supplied in the Roche Amplicor HIV-1 Monitor™ (version 1.5) kits were used as negative controls in each experiment and tested repeatedly negative for all assay methodologies used in this evaluation. RT enzyme activity and p24 antigen measurements were determined retrospectively on frozen plasma samples of all 117 samples once Amplicor HIV-1 RNA copy determinations were available.

3.3.1 Description of data obtained for a cohort of 117 plasma samples using the gold standard viral load assay and CD4 counts

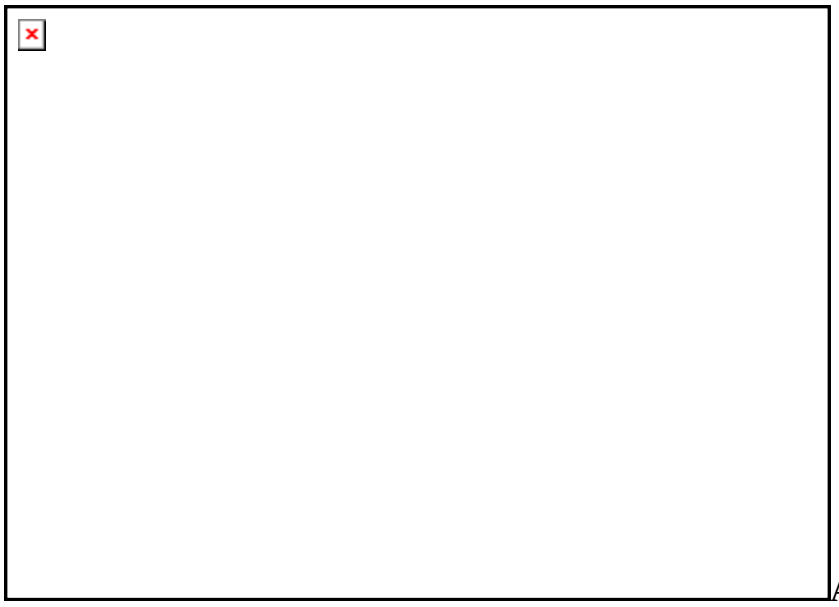
Viral load values obtained for 117 samples using RNA assay ranged from 1.7 $\log_{10}$ (50 copies/ml) to 5.9 $\log_{10}$ (750 000 copies/ml), which is within the lower and the upper limits of quantitation of the assay. Figure 3.1 A illustrates the distribution of the data within these $\log_{10}$ intervals. Approximately 40.5% of the samples tested by the RNA assay had viral load results less than 2.8 $\log_{10}$ (630 copies/ml), showing skewing of the results toward the group with lower $\log_{10}$ values. The box plot in Figure 3.1 B illustrates the range of CD4 counts for 89 samples obtained from 20 patients over six visits. A line drawn through the

median shows an increase in a median CD4 count of 186 cells/µl at visit 1 to 304 cells/µl at visit 6.

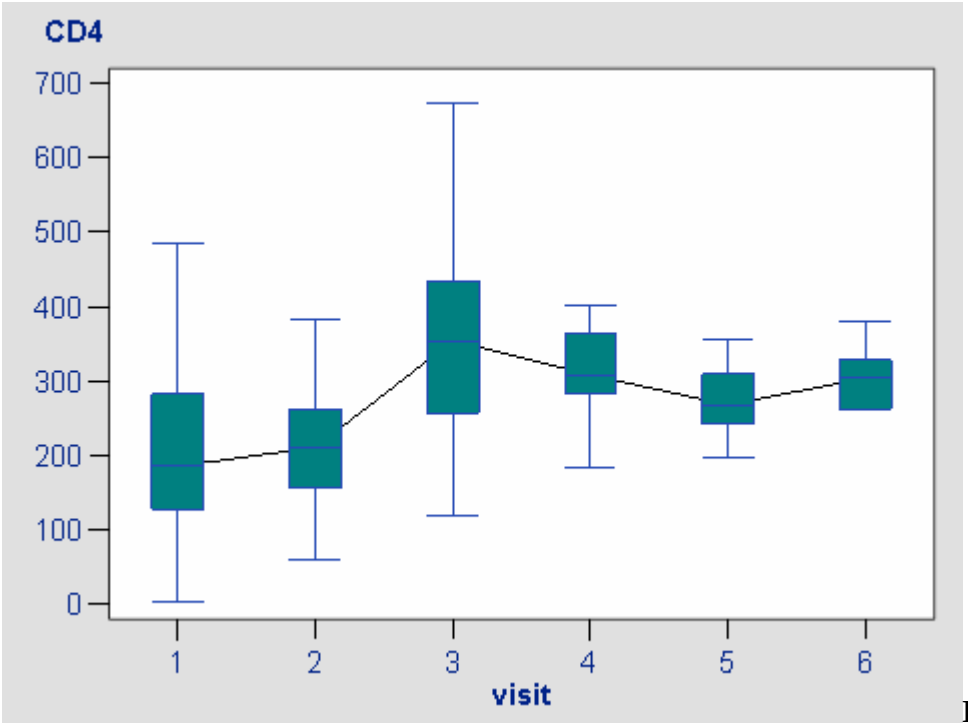
3.3.2 Comparison of the two alternative viral load assays to the RNA assay – statistical analysis

The data set obtained from these 117 samples with the two alternative methods and the gold standard HIV RNA assay were analysed using scatter plots. Figure 3.2 A illustrates a scatter plot for the p24 assay with the $\log_{10}$ p24 antigen concentration values indicated on the vertical axis and the corresponding $\log_{10}$ RNA concentrations on the horizontal axis.

On this scatter plot approximately 39% of $\log_{10}$ RNA viral load values, which cover a range of 2.5 to 5 $\log_{10}$ (316 to 100 000 RNA copies/ml), were represented by no change in p24 antigen levels between visits. In the scatter plot of the $\log_{10}$ RT level versus the RNA $\log_{10}$ concentrations in Figure 3.2 B, the lowest RNA copy number detected by the RT assay is 3.5 $\log_{10}$. Due to this quantitation cut-off level, the change in the RNA load between 50 and 3000 copies/ml was not reflected by a change in the RT level. This was represented by approximately 52% of the results. The scatter plot for the RT assay also demonstrated the ability of the RT assay to measure viral load beyond the upper limit of the RNA assay (>750 000 copies/ml). Therefore, both alternative viral load assays when compared to the gold standard (i.e. RNA assay) revealed insufficient sensitivity of quantitation at the lower viral load range. The scatter plots also showed heteroscedasticity (increasing variance with increasing values of $\log_{10}$ RNA load), which was adjusted for in the statistical analysis.

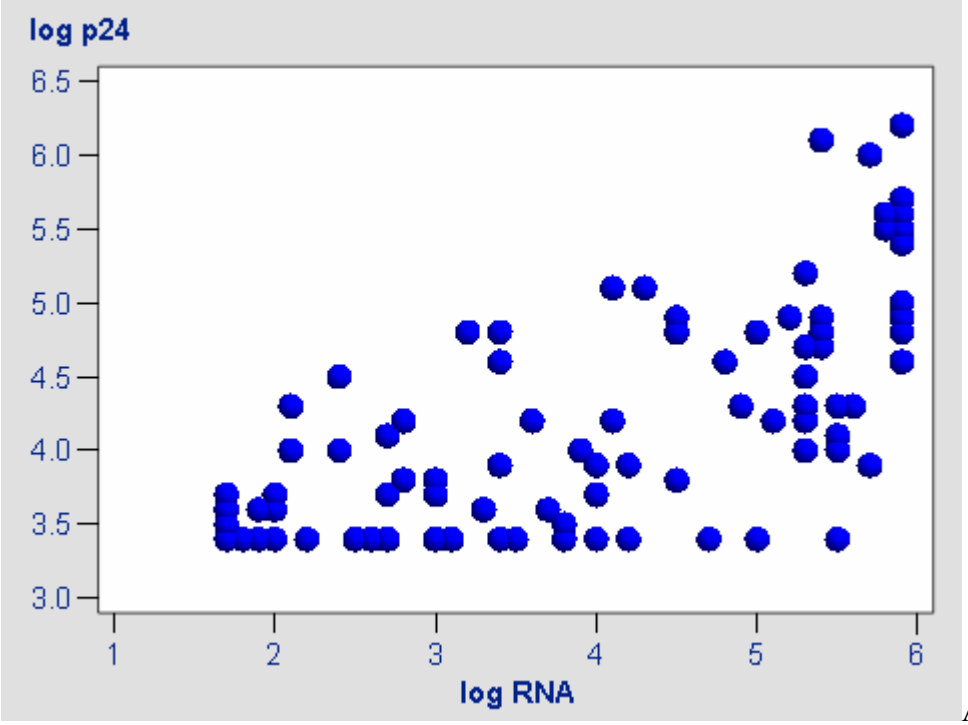


A

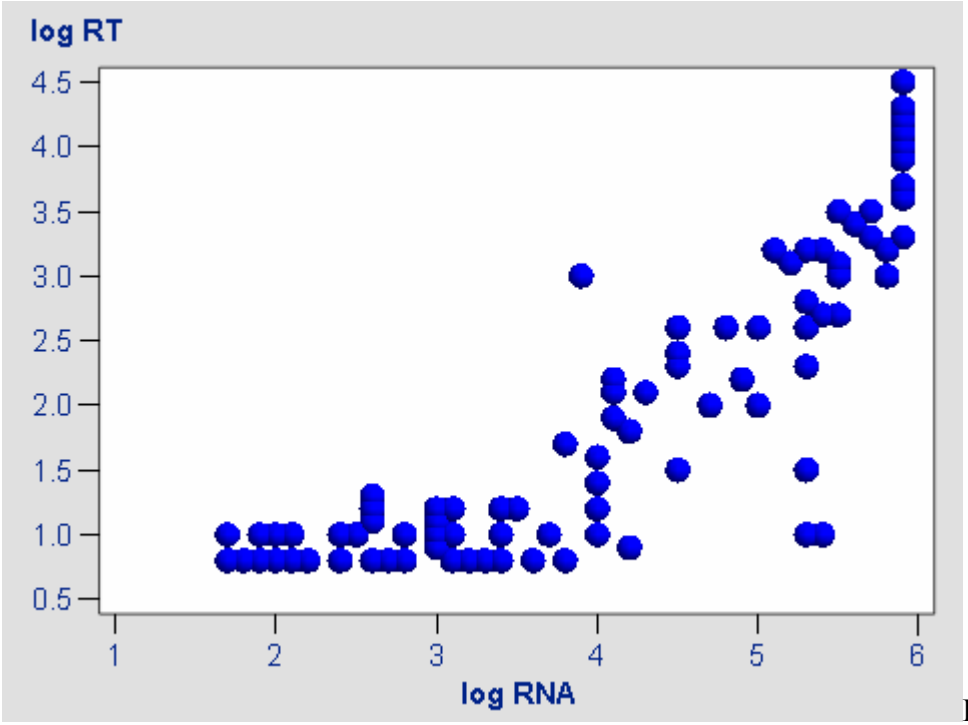


B

Figure 3.1: Distribution of log10 RNA results and range of CD4 counts (Adapted from Stevens et al., 2005a). A) Distribution of log_{10} RNA results: of the samples tested (n=117), 40.5% of samples tested had viral loads <2.8log_{10} (630copies/ml) (n=117) B) Range of CD4 counts in 20 patients over 6 visits (n=89).



A



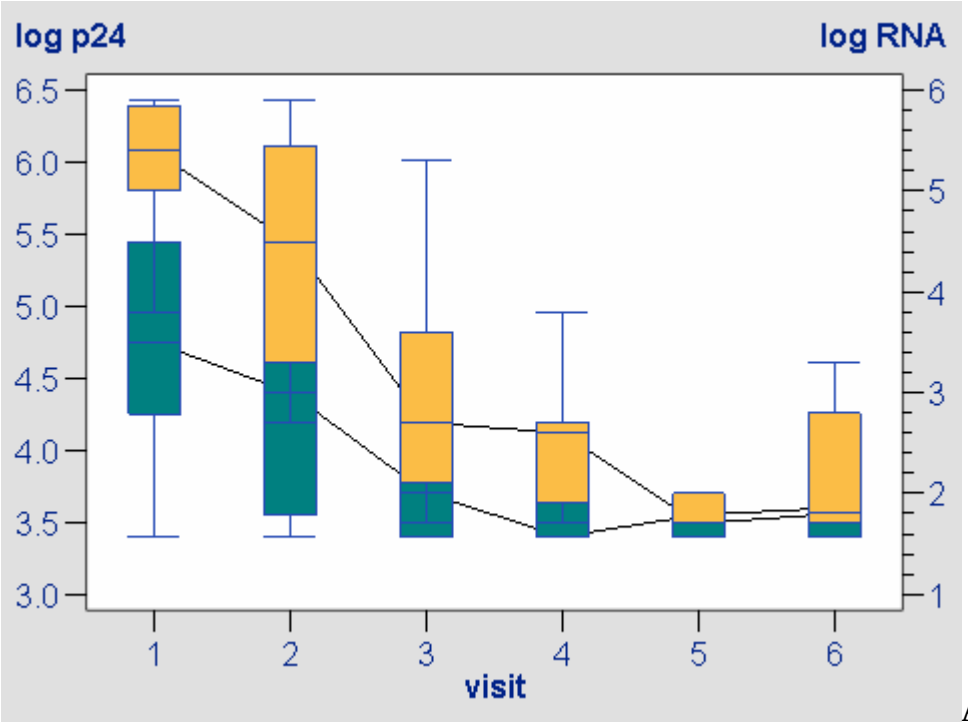
B

Figure 3.2: Scatterplots (Adapted from Stevens et al., 2005a). A) Scatterplots of $\log_{10}$ p24 level versus $\log_{10}$ RNA; and B) scatterplots of $\log_{10}$ RT vs. log RNA (n=117 samples).

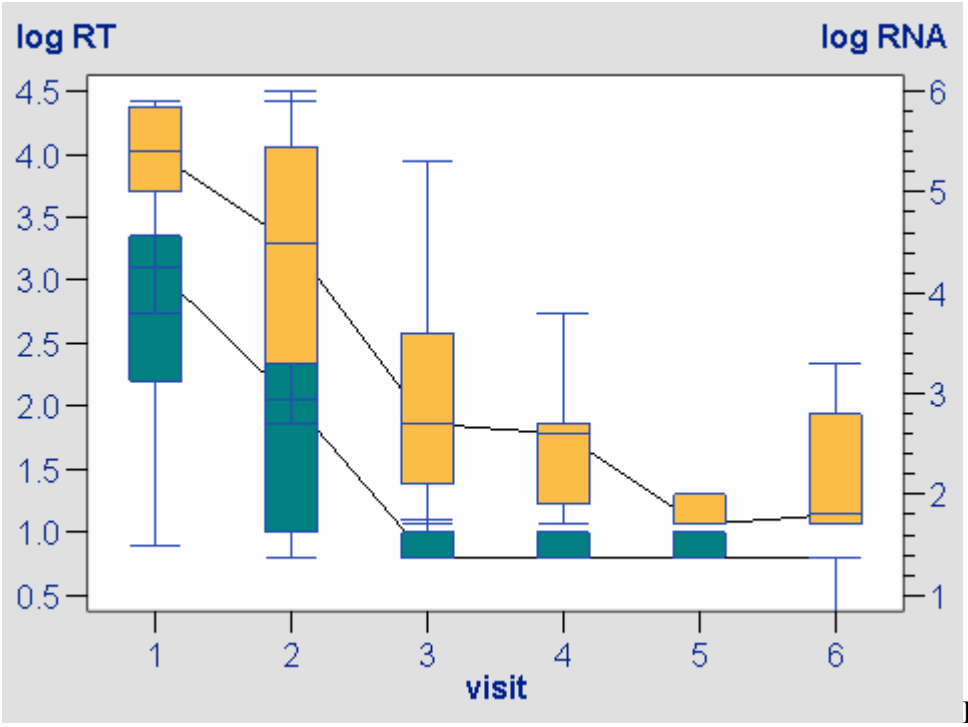
3.3.3 Analysis of patient viral load during ARV using all three monitoring assays

The mixed effect model, performed on 89 samples from 20 patients, for $\log_{10}$p24 (the regressor) and $\log_{10}$RNA (the outcome variable) showed a significant association (having adjusted for heterogeneity, replication and the effect of time). The R-square for this model equals 0.686, thus 68.6% of the variation in $\log_{10}$RNA was explained by the model. Also the slope parameter for $\log_{10}$p24 equals 1.0386, which is very close to 1 thus indicating a fair amount of agreement between the two assays. Similarly, the mixed effect model for $\log_{10}$RT (the regressor) and $\log_{10}$RNA (the outcome variable) showed a significant association (adjusting for heterogeneity, replication and the effect of time). The R-square for this model equals 0.8103, thus 81% of the variation in $\log_{10}$RNA was explained by the model. Also the slope parameter for $\log_{10}$RT equals 0.8663, which is also close to 1 but indicating less agreement between the two measures.

The box plots in Figure 3.3A and B of $\log_{10}$p24 and $\log_{10}$RT vs. $\log_{10}$RNA show the range of assay measurements on 20 patients followed over 6 visits. In 19 of the 20 patients the p24 antigen and RT serial results completely paralleled the RNA results. The concentration of p24, RT and RNA exhibited a distinct decrease in all patients after therapy initiation, with the exception of 2 patients in whom p24 and RT remained undetectable. All patients tested showed no significant viral rebound or lack of response and thus the performance of these assays in these scenarios could not be assessed. The repeated measures analyses with orthogonal polynomial contrasts have a significant linear decline in $\log_{10}$p24 ($P<0.0001$) and a curvilinear decline (quadratic trend) in $\log_{10}$RT ($P<0.0001$) and $\log_{10}$RNA ($P<0.0025$).



A



B

Figure 3.3: Box plots showing trends in viral load values over time (Adapted from (Stevens et al., 2005a). A) Schematic box plots showing $\log_{10}$ p24 and $\log_{10}$ RNA levels (p24 values = darker boxes, RNA = lighter boxes); B) Schematic box plots showing $\log_{10}$ RT and $\log_{10}$ RNA levels (RT values = darker boxes, RNA = lighter boxes). The secondary

axis versus the six visits over time was used (n = 89). A line has been drawn through the median of each visit.

All three assays were inversely correlated with CD4 T-cell counts as shown by the Spearman test (CD4 vs. RNA r = -0.336, p = 0.001; CD4 vs. p24 r = -0.541, p < 0.0001; CD4 vs. RT r = - 0.358, p = 0.0006).

3.4 Discussion

The evaluation of the p24 antigen and RT enzyme assays using 48 HIV-1 positive patients (117 plasma specimens) from South Africa suggests that these assays may provide less expensive and simpler alternatives for monitoring the response to therapy in a predominantly HIV-1 subtype C infected population. The scatterplots in Figureure 3.2 show heteroscadasticity (increasing variance with increasing $\log_{10}$ RNA loads) and insufficient sensitivity of both assays. Based on these results obtained for 117 samples, the quantitation limit for the p24 antigen assay is ~ 5 $\log_{10}$ and for the RT assay the sensitivity cut off is ~ 3.5 $\log_{10}$. The variability found for the alternative assays may be due to the fact that all three assays compared in this study measure completely different parameters of viral replication. The RT assay measures the activity of the virus-encoded enzyme, reverse transcriptase (RT), The HDp24Ag assay measures the p24 antigen either virion bound or relatively free circulating as immune complexes (Schüpbach et al., 1994) and the Roche RNA assay quantitates virion associated RNA in the blood plasma. Nevertheless, the mixed effects model shows a significant association between p24 antigen level, RT concentration and RNA viral load. Another evaluation of these alternative assays conducted in Burkina Faso, West Africa revealed good agreement between the RNA assay (Roche Amplicor) and RT activity quantitation, but poorer agreement with the p24 antigen

assay. This study also showed a similar pattern of HIV-1 viral load in the follow-up patients using the RNA and the RT activity assays (Lombart et al., 2005).Therefore, these alternative assays are more useful for the longitudinal monitoring of patients on ARV therapy rather than for a once off measurement of HIV-1 viral load. This study revealed that both assays under evaluation show a significant decline in viral load values over time in response to therapy (Figureure 3.3 A and B). In addition all 3 assays show the expected negative correlation with CD4 count (Holland et al., 2000), a conventional measure of the patient's response to therapy (Johanson et al., 2001; Schüpbach et al., 2003). The specificity of both alternative viral load assays was not addressed in this study and requires additional exploration in future evaluations.

Since this evaluation the HiSens™ HIV-1 p24 Ag Ultra assay and the reverse transcriptase (RT) enzyme activity assay have undergone further development and the new generations of these assays, especially the RT activity assay, have a ten-fold improved sensitivity. This improvement in sensitivity of the RT activity assay is achieved due to improved washing of the captured virions, an improved virion lysis procedure, doubling the amount of the template and prolonged time for RT assay (Malmsten et al., 2005; Sivapalasingam et al., 2005). It was shown that the p24 antigen assay has good reproducibility and a sensitivity limit of 100% at ≥ 30 000 copies/ml when compared to the Versant bDNA assay (version 2.0 or 3.0. Bayer Corp.) (Respess et al., 2005). The study was conducted comparing performance of the newer generation kits of both alternative assays to the Roche Amplicor assay (Jennings et al., 2005). Sensitivity limit of 400 copies/ml was demonstrated for the RT activity assay (the ExaVir™ Load assay, version 2), which is comparable to the Roche Amplicor standard assay. The RT activity assay showed excellent correlation with the Roche Amplicor assay. The same group of investigators demonstrated poorer sensitivity of

the p24 antigen assay with a limit of detection at approximately > 250 000 copies/ml (~ 5 $\log_{10}$) and lower correlation with the RNA assay (Roche Amplicor) when compared to the RT activity assay. It was also suggested that more data is required to understand the utility of the p24 antigen assay for monitoring patients on ART, since the assay measures virion-associated and non-virion-associated p24 antigen and the levels of both types of antigens may be influenced by therapy (Jennings et al., 2005).

A major consideration when evaluating these alternative assays is to estimate not only the cost but also the complexity of conducting the investigations. In this study careful attention was paid to the technical aspects of the proposed viral load methodologies. The RNA assay is automated to a large degree allowing for easier implementation and standardization. However, the equipment is sophisticated and costly and requires an appropriate laboratory design to avoid cross contamination commonly seen with PCR technology. Both alternative assays can be implemented without a specialized laboratory set-up and use less expensive laboratory equipment such as an incubator, heating block, pipettes, ELISA plate reader and washer and a computer for quantitative data analysis. Most medium tier laboratories usually possess this type of equipment already. However, the disadvantages that remain for the RT assay include (i) time taken to complete the assay for only 32-30 samples (2-3 days); (ii) the large sample volume required for analysis may pose problems in the pediatric treatment environment or in cases where a repeat test is required. Although the new generation RT assay has an improved sensitivity the technical procedure of the assay hasn't changed. The p24 antigen assay has the advantage of facilitating a higher throughput than either the RT or RNA quantitation assays. The disadvantages of this assay include: (i) the variability of the p24 antigen results for a given RNA viral load (Figureure 3.2 A) and (ii) the fact that the external virus disruption (lysis) buffer used to improve the

sensitivity of the assay is not currently commercially available. In fact, two studies conducted by different groups showed weak correlation between p24 antigen levels and viral RNA in patients on HAART when the HD p24 antigen assay was performed without the external virus disruption buffer - SNCR buffer (Bonard et al., 2003; Prado et al., 2004). By contrast, the study where the external lysis buffer was used reports on detectable levels of p24 antigen reflecting viral rebound and an inverse correlation with $CD4^+$ T cell loss during structured treatment interruption (STI) (Schüpbach et al., 2005). Recent work demonstrated that the use of the external virus disruption buffer (SNCR buffer) significantly increases detection of particle associated HIV-1 p24, thus improving quantitation of p24 concentrations in samples with medium to high HIV-1 RNA (Schüpbach et al., 2006).A shortfall of both of the alternative assays is the lack of automation which may significantly increase the person-person variation and thus affect reproducibility of these assays. Based on our experience it will require a highly skilled and experienced technologist to obtain accurate and reproducible results using these alternative viral load assays.

Full economic costing of each assay is required before implementation in any laboratory environment and should include: (i) the cost of reagents and consumables, (ii) labor, (iii) equipment and maintenance, (ix) transport of samples and fixed overheads. Preliminary estimation shows that the cost for reagents for the RT assay (per test) is approximately $10 – 20 and for the p24 antigen assay ~ $10, which is significantly less expensive than the Roche Amplicor assay (~ $75). These costs may vary substantially in each geographical region and informed decision making thus needs to be facilitated to ensure the choice of an appropriate technology is based on costs, specimen volume and skill set available. Based on the data generated in this study, both alternative viral load assays represent viable

alternatives for further exploration in certain resource-constrained environments, such as the medium tier laboratories in SA.

CHAPTER 4

Development and preliminary evaluation of a novel isothermal amplification technique for detection of human immunodeficiency virus type 1 RNA in plasma

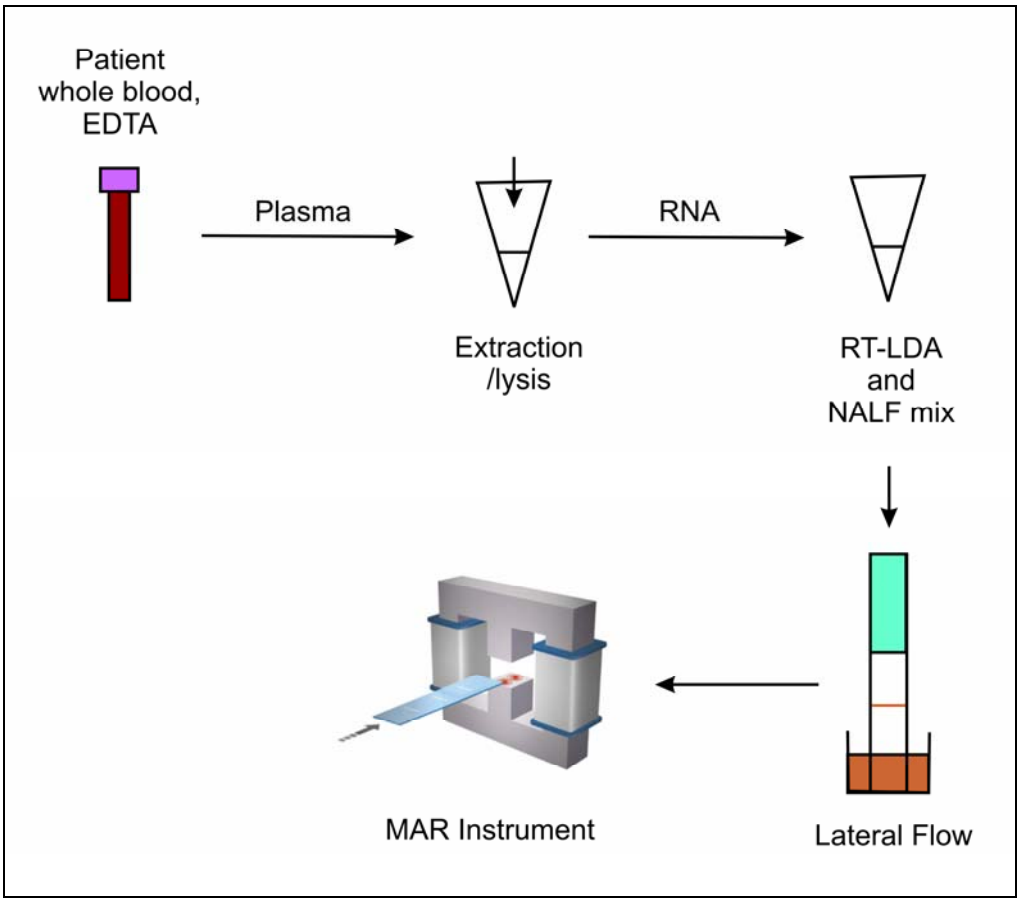
The most challenging and interesting component of this PhD project was the development of an innovative primer design named Loop Mediating Primer (LMP) that was applied to a novel isothermal amplification technique termed Reverse Transcription Loop Dependant Amplification (RT-LDA). This technique was specifically designed to amplify HIV-1 RNA for a novel NAAT based point - of - care (POC) assay for early diagnosis of acute HIV-1 infection. This chapter describes a proof - of - concept study for two versions of the LMP design and two versions of the RT-LDA technique that were optimised and evaluated using *in vitro* transcribed RNA containing 128 bases of HIV-1 *gag* gene (ES RNA). According to the POC HIV-1 RNA assay design, detection of the amplification product is performed using nucleic acid lateral flow (NALF) and superparamagnetic particles as reporter molecules. Signal acquisition from these particles is achieved using the Magnetic Assay Reader (MAR™). Detection of RT-LDA amplification product was performed using FRET hybridization probes on the LightCycler® platform. This detection format is cost effective, sensitive and sequence specific and was chosen due to the unavailability of the MAR™ instrument in South Africa. Visual detection of amplicons using dipsticks was performed at the final stage of this proof - of - concept study to confirm compatibility of this novel amplification technique with the NALF detection format.

4.1 Background

4.1.1 Assay Design

The relevance of developing a NAT based POC assay for early diagnosis of acute HIV-1 infection in the South African population was discussed in Chapter 1 of this thesis. The overall aim of this project was to develop an automated, POC assay for detection of HIV-1 RNA in plasma. The proposed POC HIV-1 RNA assay design took into account the main requirements of POC testing. In general, POC tests are rapid, require minimal training, are easy to perform and do not require a laboratory set-up. A schematic representation of the assay design is shown in Figureure 4.1. Furthermore, it is envisaged that the entire process will be performed in one closed, automated system. According to the provisional design the POC HIV-1 RNA assay includes the following steps:

- Separation of plasma from patient whole blood collected in an EDTA tube;
- Filtration of plasma into a test tube containing lysis reagents for isolation of viral RNA;
- An isothermal amplification of HIV-1 RNA using RT-LDA;
- Addition of lyophilization buffer, detection probes, and other components required for NALF;
- NALF using nitrocellulose dipsticks (NALF strips) for capture and detection of amplification product labelled with superparamagnetic beads ;
- Final detection of the captured product using a magnetic assay reader (MAR™) – signal acquisition.



Figureure 4.1: Point - of - care RNA detection assay for diagnosis of HIV-1 – assay design. Main steps: 1) EDTA whole blood collected; 2) viral RNA isolated from plasma; 3) RNA is transferred to the next tube for an isothermal amplification using a novel RT-LDA method, NALF components are added to the same test tube; 4) after RT-LDA the amplification mix is applied to a dipstick for NALF; 5) after completion of lateral flow HIV specific product is captured on a dipstick and detected by the MAR™ instrument.

This assay design combines a few novel approaches to POC diagnosis of HIV infection. In particular, the use of the Magnetic Assay Reader (MAR™) for POC HIV diagnostics is novel and was given a priority due to its technical parameters. An innovative MAR™ technology is patented by Quantum Design Inc. (QD/MBS, San Diego, USA). The MAR™ platform provides quantitative results by the detection of superparamagnetic particles as reporter elements (i.e. labels) which are bound to the analyte (Figure 4.2 A, B and Figure 4.3). The intensity of the magnetic signal (Figure 4.2 C) can be directly correlated to the amount of analyte in the sample. The platform offers sensitivity 10-1000 fold greater than visual assays. The MAR™ instrument can be miniaturized, which makes it easily adaptable for POC testing (Figure 4.2 D). MAR™ technology is compatible with well-known assay formats such as lateral flow (Figure 4.2 A) and ELISA. The POC HIV RNA test under development employs a lateral flow detection format in combination with the MAR™ platform (Figure 4.1). An initial feasibility study for this project was conducted in collaboration with British Biocell International (BBI, Cardiff, UK; http://www.bbigold.com). BBI provide expertise in lateral flow and custom particle (superparamagnetic, latex, gold colloid, etc) conjugation technologies. BBI and MagnaBioSciences, LLC, a subsidiary of Quantum Design, Inc. (QD/MBS, San Diego, USA) have formed a strategic alliance to market a feasibility service to evaluate BBI's proprietary conjugations and/or lateral flow in combination with QD/MBS MAR™ platform for a particular assay in a fast and cost-effective way. The first feasibility study aimed to evaluate probe design (Figure 4.3) proposed for the POC HIV-1 RNA test., optimise NALF conditions and estimate preliminary sensitivity of detection using the MAR™ instrument. This was performed using two structurally different synthetic single stranded DNA (ssDNA) targets (looped and linear ssDNA molecules that imitate real amplicons) containing HIV-1 sequence. Another feasibility study was initiated in parallel

and focused on developing a novel type of amplification technique suitable for the proposed POC HIV-1 RNA test.

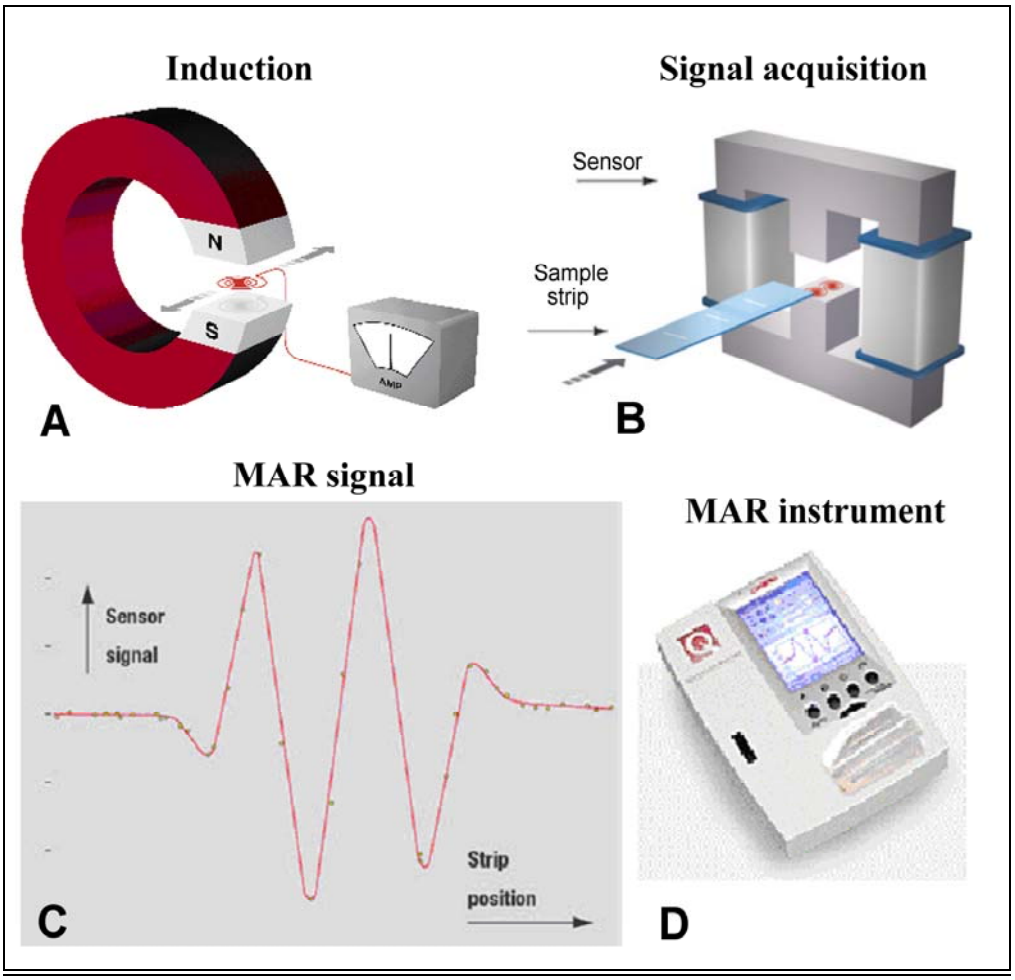


Figure 4.2: Magnetic Assay Reader (MAR™). A and B: Induction of a magnetic signal by exposing the superparamagnetic particles to a magnetic field and acquisition of the signal by the MAR™ instrument; C: A magnetic signal registered by the MAR™ from the labelled analyte captured on the NALF dipstick; D: A hand held version of the MAR™ instrument. (Images were adapted from: http://www.qdusa.com/index.html).

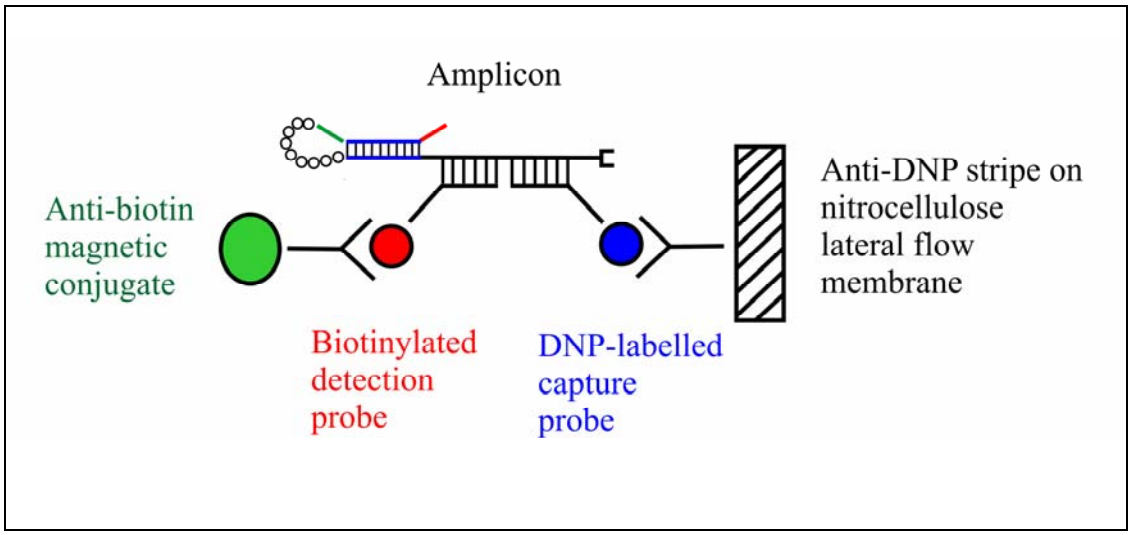


Figure 4.3: Detection of amplicons using lateral flow dipsticks and superparamagnetic conjugates. The detection probe labelled with biotin and the capture probe labelled with DNP bind to the partially looped ssDNA amplicons. Anti-DNP antibodies that are striped on the dipsticks capture the entire "amplicon/probes" hybridisation product. Anti-biotin antibodies labelled with the superparamagnetic conjugates bind to the biotin on the detection probe allowing visual and/or MAR™ based detection of the product captured on the dipsticks.

4.1.2 Novel isothermal amplification technique for POC HIV-1 RNA test

The term "isothermal' indicates that the entire amplification process is performed under one constant temperature. Even though, many isothermal amplifications use an initial denaturation at high temperature or an initial reverse transcription (RT), ligation steps at a lower temperature, compared to PCR amplification, there is no temperature cycling. Therefore, an isothermal amplification does not require a thermocycler and it can be performed in a simple heating block. This feature makes an isothermal amplification approach adaptable to the POC testing format. There is a variety of published and commercially available methods for isothermal amplification of nucleic acids. Approved isothermal amplification techniques that are most commonly used for diagnostics may be

classified into two major groups: signal amplification and target amplification. Signal amplification methods include: branched DNA (b-DNA) amplification (see Chapter 1, section 1.2), hybrid capture (Wick, 2000), cycling probe technology – CPT (Fong et al., 2000; Modrusan et al., 2000), rolling circle amplification – RCA (Blab et al., 2004; Christlan et al., 2001; Zong et al., 2001), ramification amplification (Zong et al., 2001) and circle-to-circle amplification (Dahl et al., 2004). Among widely used target amplification methods are: NASBA (see Chapter 1, section 1.2), transcription mediated amplification - TMA (Emery et al., 2000), strand displacement amplification - SDA (Hellyer et al., 1999; Nadeau et al., 1999; Nycz et al., 1998; Pilcher et al., 2004a; Spargo et al., 1996; Walker, 1993; Walker et al., 1995; Walker et al., 1992; Walker et al., 1996; Walker et al., 1994), loop-mediated isothermal amplification - LAMP (Notomi et al., 2000; Thai. et al., 2004) and Helicase - dependant isothermal DNA amplification - HDA (Vincent et al., 2004). The techniques listed above differ in their design and technical characteristics such as amplification efficiency and type of amplicons produced. Based on the proposed design for the POC HIV-1 RNA test (Figure 4.1), the amplification technique has to meet certain requirements. Firstly, it has to provide great power of amplification so that even low concentrations (3-4 $\log_{10}$ copies/ml) of virus can be amplified sufficiently in a short period of time. Secondly, an amplification method has to convert viral RNA into the short (maximum 200 bases), ssDNA or RNA amplicons in order to be readily compatible with the lateral flow detection format. To date, none of the available isothermal amplification methods fulfils both requirements at the same time. Signal amplification techniques are usually linear and take at least a few hours to provide sufficient sensitivity. Amplicons produced with these techniques are either long stretches of DNA (e.g. RCA) or complex, “branched” molecules (e.g. bDNA). Isothermal target amplification techniques which utilize the concept of strand displacement amplification (e.g. SDA, LAMP) show greater

efficiency than the techniques based on RNA transcription (e.g. NASBA, TMA). SDA and LAMP methods have good sensitivity, but in their existing format these methods cannot be used for the proposed POC HIV-1 RNA test since final amplification products are double stranded DNA (dsDNA) fragments and long stretches of dsDNA respectively. In addition, to accelerate the development process, the use of commercially available enzymes is desirable. SDA provides great power of amplification that would be suitable for the designed POC HIV-1 RNA test. During the exponential, cycling phase of SDA the typical target doubling time is 20-30 seconds and thus a 10 billion-fold amplification of specific targets can be achieved in less than 15 minutes (Spargo et al., 1996). Therefore, to achieve significant amplification it was decided to utilize the combination of enzymes used in SDA as well as the concept of including a nickable site in the primer sequences for a novel amplification. Figure 4.4 illustrates the mechanism of strand displacement amplification. SDA makes use of two enzymes – *BsoBI* restriction enzyme and *Bst* DNA polymerase that possesses the ability to synthesize new DNA strands and simultaneously displace the existing strand. In the case where RNA is used as a template the RT step is performed by the third enzyme – AMV (Avian Myeloblastosis Virus) reverse transcriptase (RT – SDA). Both SDA and RT-SDA consist of two phases (target generation and exponential target amplification) and employ four primers (a pair of bumping primers and a pair of primers containing a nickable site; see Figure 4.4). During the second, cycling phase of SDA dsDNA amplicons accumulate exponentially (Hellyer et al., 1999; Nadeau et al., 1999; Nycz et al., 1998; Spargo et al., 1996; Walker, 1993; Walker et al., 1995; Walker et al., 1992; Walker et al., 1996; Walker et al., 1994).

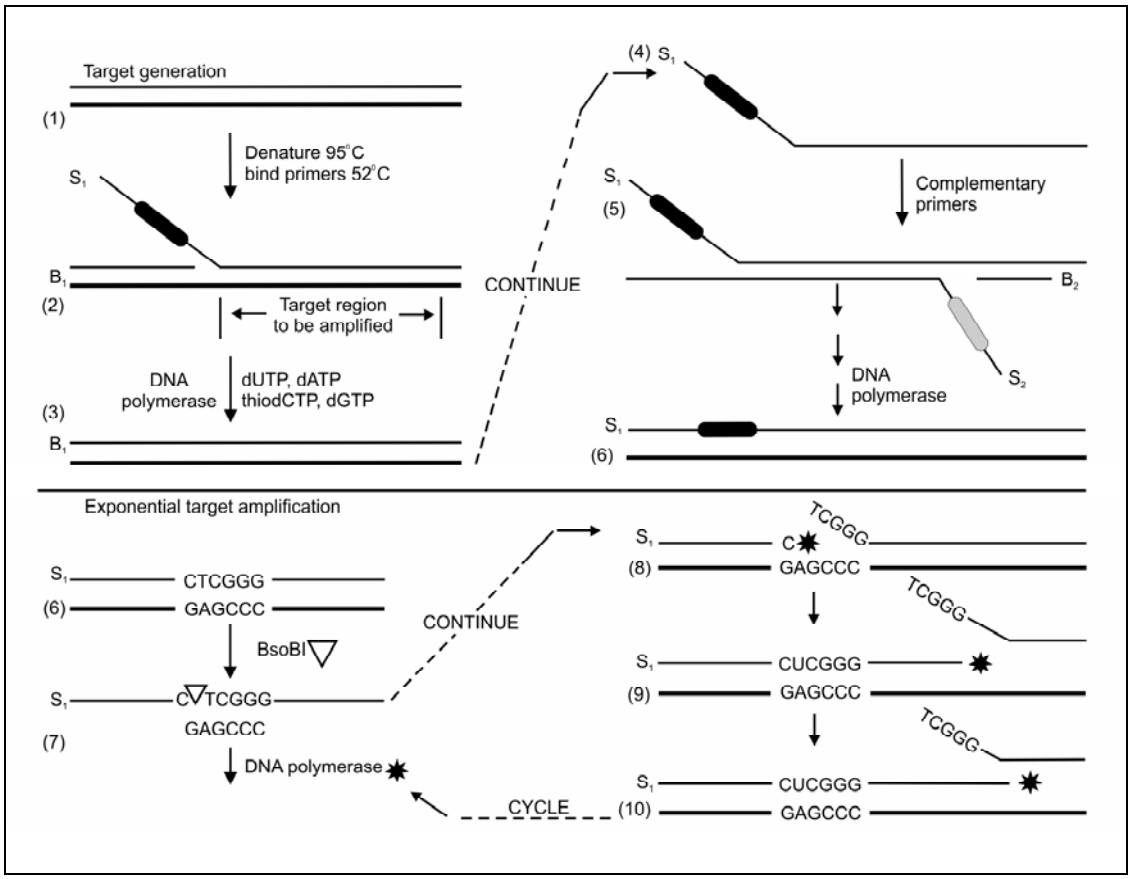


Figure 4.4: Strand Displacement Amplification (SDA) (Adapted from Schweitzer and Kingsmore, 2001). In the target generation phase (top panel) of SDA, a dsDNA target (1) is denatured and hybridized with two primers (2). One primer (B_1) is designated as a 'bumper' primer, and the other primer (S_1) contains a *Bso*BI restriction enzyme sequence 5′ to the target binding region. The B_1 (3) and S_1 (4) primers are simultaneously extended by the thermostable enzyme *Bst* DNA polymerase in the presence of thiolated dCTP. Extension from the bumper primer displaces the S_1 extended product, which can then hybridize to the opposite strand primers, B_2 and S_2 (5). Extension of both of these primers produces species 6, which is utilized in the exponential target amplification phase of the reaction (lower panel). The strand that has been extended from the S_1 primer is nicked by *Bso*BI (7), but the complementary strand is refractory to cleavage owing to the presence of a thiolated dCTP within the restriction site. DNA polymerase binds to the nick and begins synthesis of a new strand while displacing the downstream strand (8—10). This recreates the double-stranded species 7, and the process repeats. The displaced strands bind to opposite strand primers, thus producing exponential amplification.

The design of a novel isothermal amplification technique - RT-LDA, envisages converting HIV-1 RNA into short, ssDNA molecules (Figure 4.5) with high efficiency. In summary the characteristic features of this technique and differences from SDA are described below:

- RT is performed using only one HIV-1 sequence specific primer and a type of AMV enzyme that has both RT and RNase H activity (H^+). No bumping primers are used for RT-LDA as opposed to conventional SDA. Instead, cDNA (anti-sense sequence) is released from the initial RNA/cDNA complex due to RNase H activity of AMV RT, which also makes the method different from SDA (Figures 4.6 and 4.7);
- RT-LDA is achieved mainly due to the innovative primer design - LMP. One of the primers has a 5' overhang containing a sequence complementary to a sequence of a DNA strand extended from this primer (Figure 4.5). As a result of this primer modification the displaced amplicons assume a "loop" conformation at their 3' end. This "looped" structure prevents the displaced amplicons from binding to a complementary primer and thus preserves their single stranded structure;
- Due to LMP design this amplification technique (RT-LDA) does not have an exponential, cycling phase, which is characteristic for conventional SDA (Figure 4.4);
- Final amplification product generated with RT-LDA is represented by partially "looped", ssDNA amplicons (Figure 4.5; 4.6 and 4.7) as opposed to dsDNA amplicons produced by SDA. A characteristic feature of this isothermal amplification is the generation of partially looped" ssDNA amplicons, which is reflected in the name of this technique – **r**everse **t**ranscription **l**oop **d**ependant **a**mplification (RT-LDA).

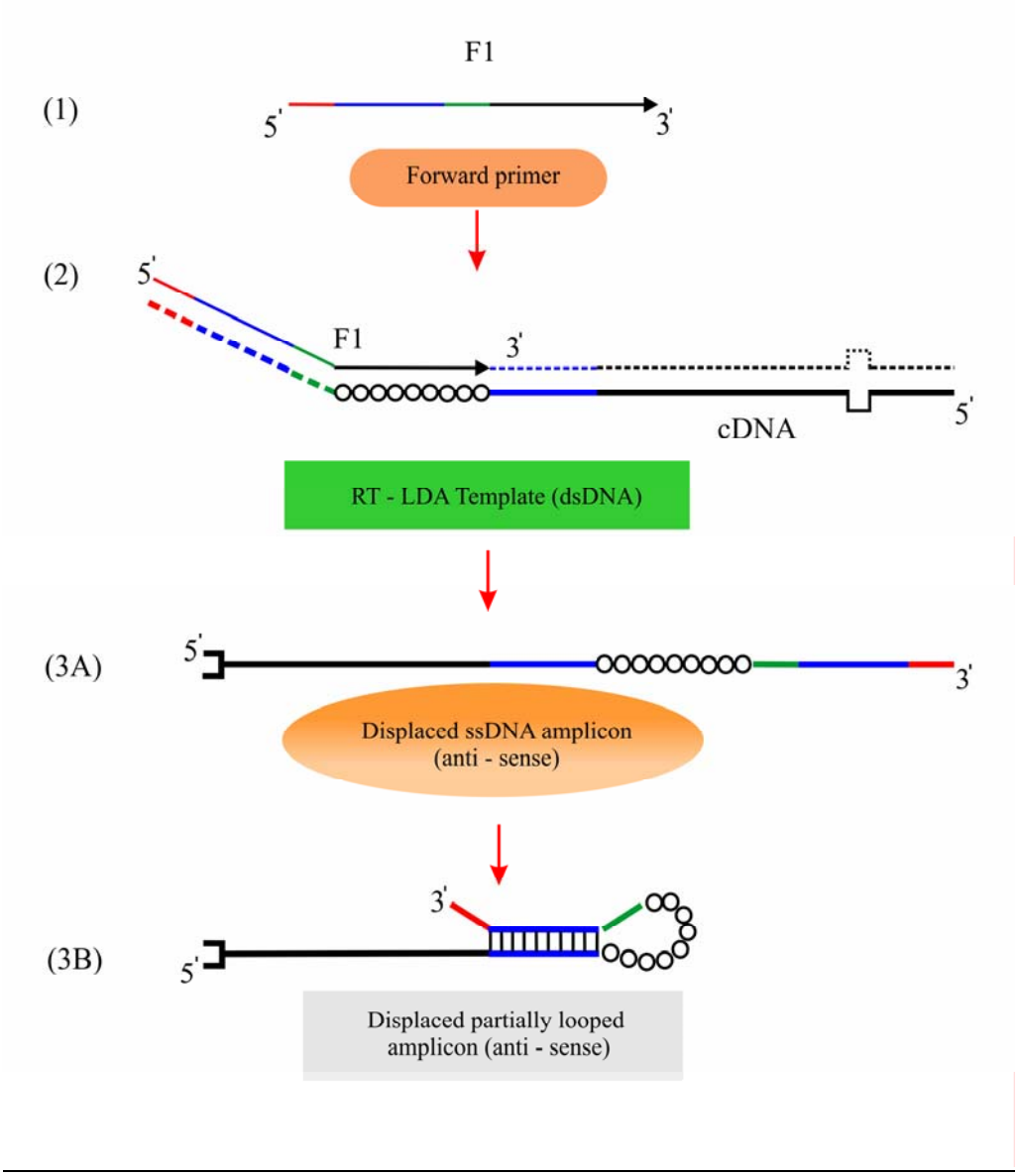


Figure 4.5: Loop Mediating Primer (LMP) design for RT-LDA.

1) Different parts of a primer that have different functions are colour coded. **Red** - a short artificial (i.e. non-target specific) sequence that serves to prevent a displaced amplicon from self-priming; **blue** - a sequence complementary to a strand extended from this primer (so called "loop" overhang); **green** - a short artificial sequence that forms a link between the "loop" overhang and a forward primer; **black** - HIV-1 sequence specific forward primer. 2) Forward primer with an overhang (parts indicated in red, blue and green) binds to the anti-sense cDNA strand and gets extended. Extension of a forward primer produces a dsDNA target. An overhang sequence also gets extended by *Bst* polymerase from the 3' end of the cDNA strand. 3A) Anti-sense strand gets displaced from the nickable site (similarly to SDA, see Figure 4.4).The displaced anti-sense strand, which now can be

termed an anti-sense ssDNA amplicon contains a sequence complementary to an overhang of a forward primer. 3B) The two complementary sequences within one amplicon are in close proximity and the 3’ end of the anti-sense amplicon folds forward and hybridizes to its complementary sequence (indicated in blue). As a result a partially “looped” ssDNA amplicon is formed. The “loop” structure at the 3’ end of an amplicon prevents it from binding to another forward primer and from getting converted into a dsDNA target. Thus, most of the displaced anti-sense amplicon remains single stranded.

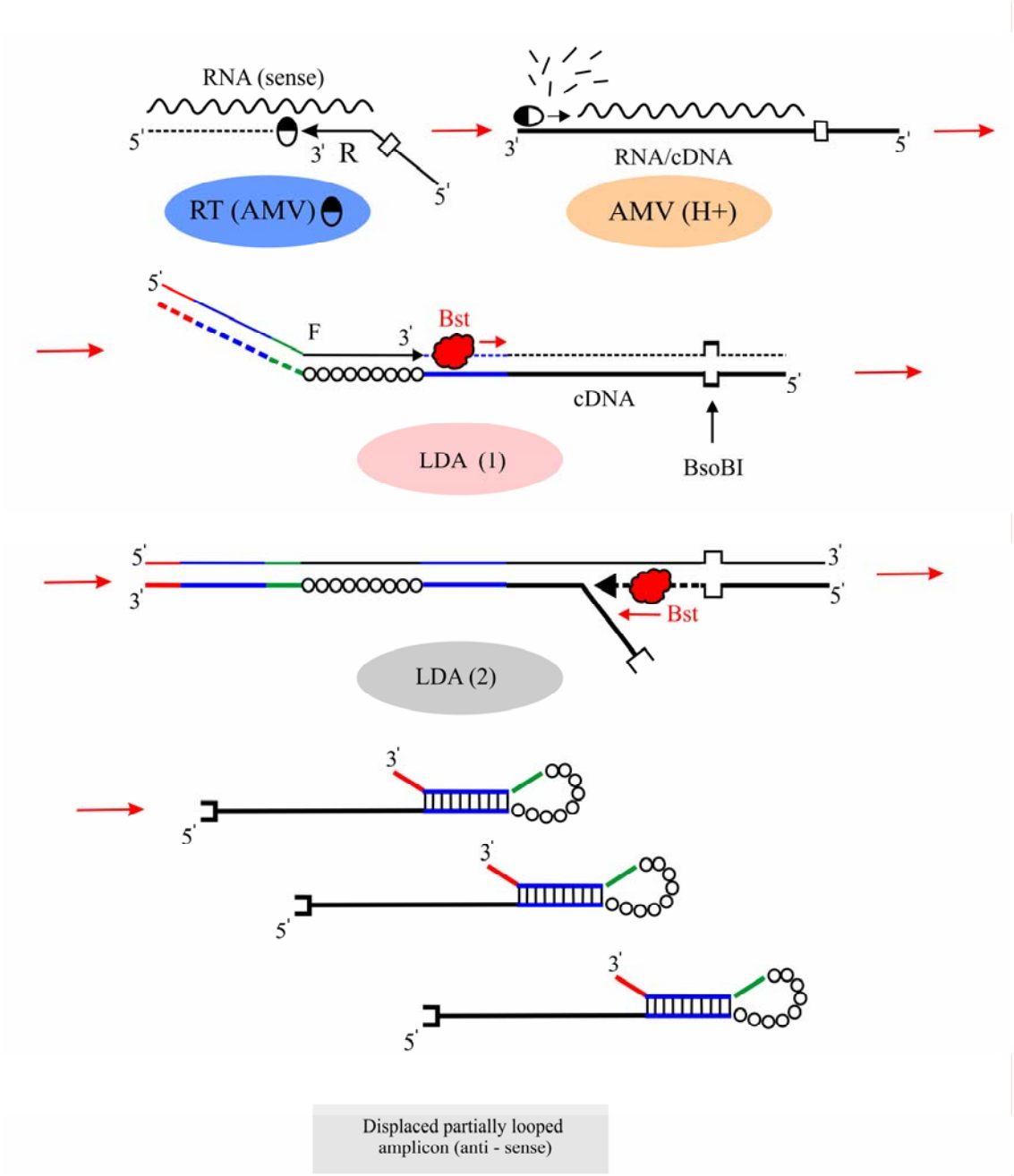


Figure 4.6: RT-LDA - design for linear amplification.

HIV-1 RNA is an initial template for amplification. A reverse primer (R) comprises of three regions, starting from the 5' end they are as follows: an artificial (random) sequence, sequence, recognition site (so called nickable site) for the restriction enzyme *Bso*BI (indicated as a box), and HIV-1 sequence specific (reverse) primer. Reverse primer (R) binds viral RNA and gets extended by the RT activity of AMV reverse transcriptase enzyme (RT). A double stranded RNA/cDNA hybrid is formed. The RNase H activity of

AMV digests viral RNA in the hybrid leaving only cDNA (anti-sense sequence). (AMV H^+). A forward primer (F) (see Figure 4.4 for the detailed description) converts cDNA into dsDNA target (LDA 1). Due to the addition of chemically modified dCTPs (dCTP - αS) in the reaction mix the double stranded *Bso*BI recognition site is hemithiolated and only gets nicked by the restriction enzyme (see also Figure 4.3). *Bst* DNA polymerase, which also has strand displacement ability, extends the 3'-end of a newly formed nick and at the same time displaces the existing, anti-sense DNA strand (LDA 2). During this process of DNA strand extension/displacement the *Bso*BI nickable site gets regenerated and can be nicked over and over again, thus producing a large number of "looped" anti-sense ssDNA amplicons. The displaced amplicons assume a partially "looped" structure due to a carefully designed overhang of a forward primer, as described in Figure 4.4. Formation of partially "looped" amplicons prevents binding of the forward primer to the anti-sense amplicon and thus prevents the entire amplification process from cycling. The RT-LDA design illustrated in this figure provides for the accumulation of partially "looped" ssDNA anti-sense amplicons in a linear fashion.

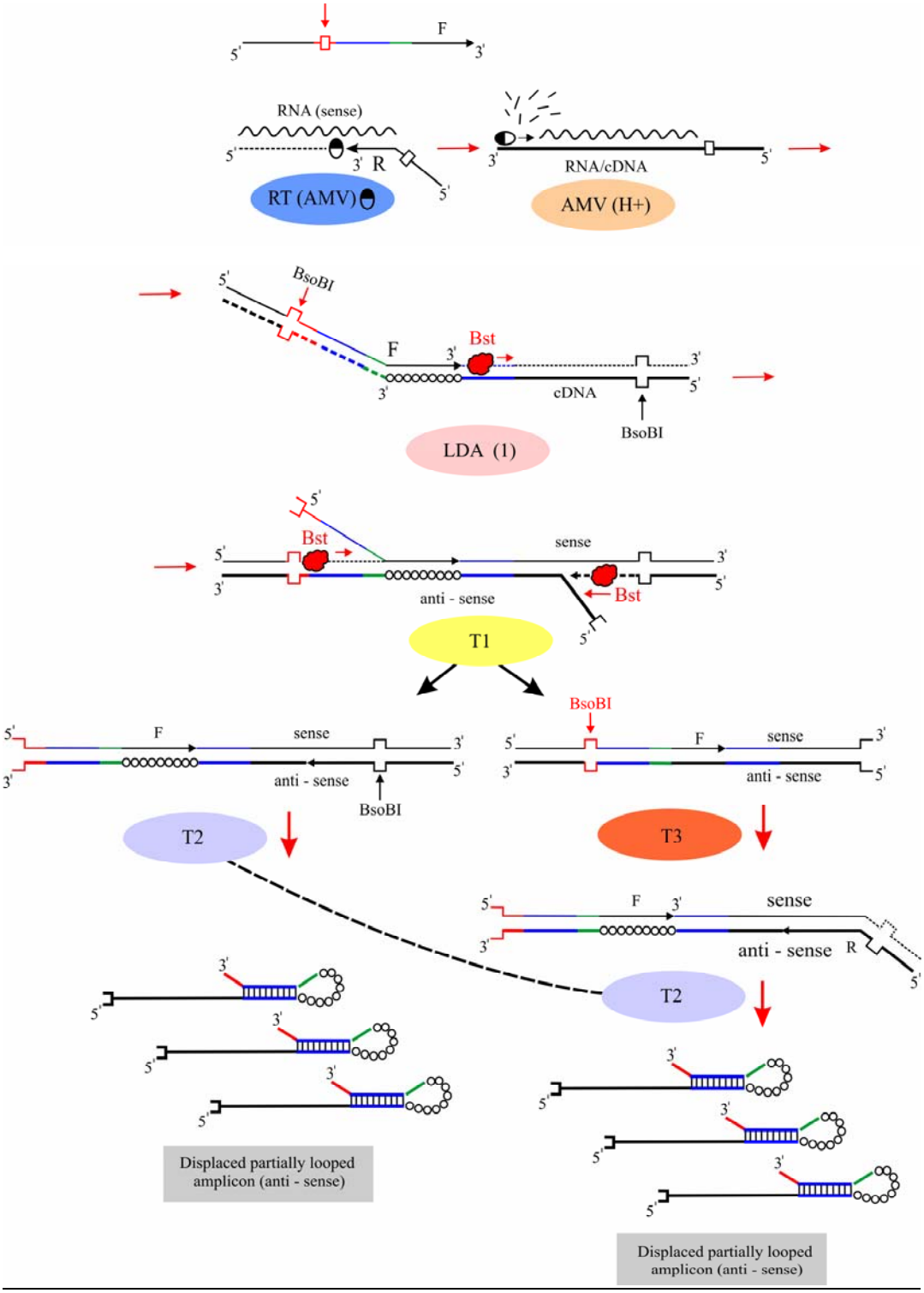


Figure 4.7: Semi-cycling RT-LDA.

Conversion of the initially designed linear amplification technique into semi-cycling was possible due to another novel design applied to the forward primer. Similarly to the original primer design (Figure 4.5) the new design includes an HIV-1 specific forward primer (**black**) with a 5' overhang, but the structure of the overhang is different. From the

5’ end the primer overhang contains” an artificial” sequence (**black**), *Bso*BI recognition site (**red box**) and a sequence complementary to an amplicon (“loop” sequence) (**blue**) and a very short linking sequence (**green**). The RT step for the semi-cycling amplification is identical to the linear amplification (Figure 4.6). A modified forward primer that binds to cDNA (anti-sense) is extended by *Bst* polymerase and as a result a dsDNA target is formed (T1). The strands complementary to the *Bso*BI restriction site are generated during the primer/strand extension and thus contain modified dCTP - αS (thiolated), which prevents *Bso*BI from cutting dsDNA. Instead, only the unmodified nickable sites (in all targets - T1, T2 and T3) from the primers get nicked (single stranded nick) by the enzyme. T1 dsDNA has two nickable sites – one on a sense strand (forward primer; red box) and another one on an anti-sense strand (reverse primer; black box). Both nickable sites are recognized and nicked (cleaved) by *Bso*BI restriction enzyme. Next, the 3’- end of each nicked strand (sense and anti –sense) is extended by *Bst* DNA polymerase and the existing strands are displaced. The displaced sense strand forms a partially “looped” amplicon that has a sequence complementary to a reverse primer. The reverse primer binds this sense amplicon and converts it into dsDNA target (T2). T2 has only one *Bso*BI restriction site and a process on nicking and strand displacement/ polymerization occurs on the T2 target in a similar way as described above. A cascade of processes between formations of T1 and T2 represents a linear phase of a RT-LDA. The displaced anti-sense amplicons have a sequence at their 3’- end which is complementary to the “loop” overhang of a forward primer. Thus, once displaced these amplicons assume a partially “looped” structure. The anti-sense strand, which is displaced from T1 possesses a 3’ sequence complementary to the entire forward primer and thus a forward primer binds to the anti-sense amplicon. Extension of a forward primer converts the anti-sense strand into dsDNA target (T3). Target T3 produces “looped” sense amplicons similarly to T1. These amplicons bind to the reverse primers which lead to the formation of dsDNA targets identical to T2. The cascade of processes, from formation of T1 to T3 and then to T2, represents a semi-cycling phase of this amplification technique. T2 generated during a semi-cycling phase also keeps producing “looped” ssDNA anti-sense amplicons. During this type of amplification both linear and semi-cycling phases of RT-LDA occur simultaneously and generate large amounts of the final amplification product - partially “looped” ssDNA anti-sense amplicons. The semi-cycling design provides for at least a 3-fold increase in magnitude of amplification.

Two versions of the RT-LDA design were made and evaluated: the initial design for linear amplification and the final design for the semi-cycling amplification technique. Figures 4.6 and 4.7 provide a detailed description of the linear and semi-cycling RT-LDA designs, respectively. Both designs produce the same final product – partially "looped" ssDNA amplicons with anti-sense sequence, but the amount of final RT-LDA product is greater in the semi-cycling technique. The sole difference between linear and semi-cycling RT-LDA is in the design of a forward primer (Figure 4.7 A). This type of primer design leads to partial cycling of the reaction via formation of 3 types of dsDNA targets. Semi-cycling RT-LDA includes two simultaneously occurring phases: linear and semi-cycling phase (Figure 4.7 B). The second design for semi-cycling RT-LDA was made at the later stage of the assay development in an attempt to improve the power of amplification of this technique.

The main challenge of this project was in obtaining experimental proof for the LMP design. The exact parameters (e.g. length of an overhang, etc) for a novel type of primer with a "loop" overhang had to be established experimentally. The "loop" structure must be sufficient to preserve the single stranded amplicons and at the same time it must not interfere with extension of the primer. Reaction conditions of RT-LDA suitable for maintaining four types of enzymatic activities also required optimization.

4.2 Materials and Methods

4.2.1 Isothermal Amplification - RT-LDA

4.2.1.1 Primers

Primers were designed for a sequence of an *in vitro* transcribed RNA that represents an external quantitation standard (ES RNA) used for the LightCycler® viral load assays – the FRET assay and the LUX assay (see chapter 2). The ES RNA molecule includes a 128 bp region of HIV-1 gag gene (Figure 2.5). Primer names were assigned based on when the primer was designed during the development (e.g. forward primer named "Fw#1" was the initially designed primer; Fw #4A – represents the final primer design). Sequences of the primers used for RT-LDA reactions are as follows (HIV specific sequence of the primers is underlined):

Forward #1
5' – AACAGCCTCC TCATTGATTG TATCTTTTAA CATGAAACAT CAAGCAGCCA TGCAAAT – 3' (57 mer)

Forward #1A
5'– AGA ATT GAT TGT ATC TTT TAA CAT ACA TCA AGC AGC CAT GCA AAT – 3'
(45 mer)

Forward #1B
5' – ACC TGT ATC TTT TAA CAT ACA TCA AGC AGC CAT GCA AAT - 3' (39 mer)

Forward #2
5' – GATATTCTGC AGGAAATACA ATCAATGAGG AGGC T – 3' (35 mer)

Forward #3
5' – GAG GCA GCC TCC TCA TGC AAA TGT TAA AAG ATA CAA TCA AT- 3'
(41 mer)

Forward #3 (a "new" version) - #3A

5'- TCT CC TCC TCA T ACT TGT TAA AAG ATA CAA TCA AT- 3' (35 mer)

Forward #1C

5' – GGC C TTT TAA CAT AGA ACA TCA AGC AGC CAT GCA AAT - 3' (37 mer)

Forward # 4A (a version of #3A with the *Bso*BI nickable site (blue))

5'- AAC CTA TCC GGA CAA CGA TAA ***CCC GGG*** CC TCC TCA T ACT TGT TAA AAG ATA CAA TCA AT- 3' (59 mer)

Reverse #R1 (blue – *Bso*BI nickable site)

5'- GTCGACTTGC ATGCATGT ***CTC GGG*** TGTCAC TTCCCCTTGG TTCT CTCA – 3'

(48 mer)

4.2.1.2 RT-LDA reaction conditions

RT-LDA amplification reactions were performed in a total volume of 50 µl comprising of 20 µl of template RNA and 30 µl of reaction mix. The reaction mix was assembled as follows: 35 mM Potassium phosphate buffer (K_1PO_4; Appendix B), 5% v/v dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), 4% v/v glycerol (Promega, Madison, WI, USA), 5 µg acetylated bovine serum albumin (AcBSA; Promega, Madison, WI), 0.8 mM 2'-deoxycytidine 5'-O- (1- thiotriphosphate) (dCTP - αS; Amersham Biosciences, Piscataway, NJ, USA), 0.6 mM dUTP, 0.2 mM each dATP and dGTP (Promega, Madison, WI, USA), 7.5 mM magnesium acetate ($MgOAc_2$; Sigma-Aldrich, USA), 7.5 units AMV reverse transcriptase (USB Corp., Cleveland, Ohio, USA), 32 units *Bso*BI restriction enzyme (New England BioLabs Inc., USA), 20 units *Bst* DNA polymerase (New England BioLabs Inc., USA), 1µg T4gp32 ssDNA binding protein (USB Corp., Cleveland, Ohaio, USA), forward and reverse primers 0.75 µM each. All reaction components and RNA template were mixed and incubated at 53 ºC for 1 hour using a heating block (Labcon,

South Africa). Synthetic *in vitro* RNA (ES RNA) described in chapter 2, section 2.2.2, was used as a template for RT-LDA reactions.

4.2.1.3 Detection of RT-LDA amplification product using FRET hybridization probes and Nucleic acid lateral flow (NALF)

Detection of RT-LDA product using FRET hybridization probes and the melting curve analysis was performed using the LightCycler® platform, version 1.2 and the software version 4 (Roche Applied Science, Mannheim, Germany). This detection format was selected since it provides sequence specific detection and thus confirmation of the amplification product. The FRET probe format is also more rapid and allows better differentiation between dsDNA targets (e.g. T1-T3; Figure 4.7) and ssDNA amplicons than agarose or polyacrylamide gels. FRET hybridization probes were designed for the anti-sense amplicons with the following sequences:

Ampi/FRET 2032:
5' – TAG AAT ACA TCC AGI ICA TGC GG – Fluorescein – 3'
Ampi/FRET 2033:
5'- LCRed -640 – CCT IIT GCA CCA GGC CTA ATG AG – p – 3' (I = Inosin)

Probes were manufactured by Metabion International AG (Germany). Reconstituted probes, each at 100 µM concentration, were kept at -20 ºC until use. The post amplification detection using FRET probes was performed in a 30 µl volume using 27 µl of amplification reaction and 0.5 µM of each probe. The profile of the melting curve analysis was as follows: 95ºC for 5 s, 40ºC for 1 min and gradual heating up to 85-90ºC with 0.1ºC/s ramping rate and continuous fluorescent signal acquisition. Melting curves were viewed using a combination of channels -640/Back530.

Reagents for nucleic acid lateral flow, including buffers, magnetic conjugate, nitrocellulose DNP striped half dipsticks, capture and detection probes were provided by British Biocell International (Cardiff, UK). Sequences of the LF probes are as follows:

Ampi 2032 – Detection probe (two inosines):
5' Biotin TCTGC ACA TCC AGI ICA TGC GGG – 3'
Ampi 2033 – DNP – capture probe (two inosines):
5' – GCC TII TGC ACC AGG CCT – DNP- 3'

The positive control for NALF detection was also supplied by BBI. The positive control (ampi29) was made to imitate RT-LDA amplicons. Ampi29 represents a synthetic ssDNA molecule that has a partially looped "structure" and the sequence similar to the real amplification product. NALF detection was performed according to the protocol developed by BBI (proprietary) during the feasibility study for the POC HIV RNA assay.

4.2.2 Conventional PCR for detection of RT - LDA product

PCR was performed in a 50 µl volume using 25 µl of ReadyMix™ Taq PCR reaction with $MgCl_2$ (Sigma, St. Louis, Missouri, USA), 0.5 µM forward and reverse primer, 15 µl of template (diluted RT-LDA product) and PCR grade water from the kit (Sigma). Different primers were used for different experiments and the exact combinations of forward and reverse primers used for particular experiments are described in section 4.3.
PCR was performed using the MyCycler™ thermal cycler (BIO-RAD Laboratories, Inc., USA) according to the following profile: initial denaturation at 95ºC for 3 min and than 40 cycles of amplification with denaturation at 95ºC for 30 s, annealing at 52ºC for 30 s, extension at 72ºC for 30s and final extension at 72ºC for 7 minutes.

Detection of PCR product was performed using high resolution MetaPhor® gels (Parry et al., 2001; Tatt et al., 2000) and conventional agarose gel electrophoresis. Fifteen microliters of amplification product were mixed with 5 µl of the gel loading dye (Fermentas UAB, Lithuania) and loaded onto the 1-2% MetaPhor® agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) gel prepared with 1xTBE buffer (diluted from 10xTBE stock; Fermentas UAB, Lithuania) and 3 µl of ethidium bromide stock solution (10 mg/ml; Sigma –Aldrich., St. Louis, Missouri, USA) per 100 ml of gel. Agarose gel electrophoresis was performed at 100 V for 1-2 hours. Size verification of the product was performed by running 10 µl of the O'GeneRuler™ 50 bp DNA ladder (Fermentas, UAB, Lithuania) along side the sample. The gel was viewed under the UV light.

4.2.3 Sequencing of RT - LDA product

PCR product amplified using the RT-LDA reaction (see section 4.2.1.3) was used as a template for sequencing. Prior to sequencing the PCR product was excised from the MetaPhor® gel and purified using the MinElute™ Gel Extraction kit (Qiagen, GmbH Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 10 µl of the elution buffer supplied in the kit.

The sequencing reaction was performed with the Big Dye® Terminator version 3.1 cycle sequencing kit from Applied Biosystems (Foster City CA, USA) according to the manufacturer's instructions, on the ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems). Primers used for sequencing are specified in the "Results" section (section 4.3) when describing particular experiments. Sequence data analysis was performed using the Sequencing Analysis program, version 3.3 (Applied Biosystems) and assembled using

Sequencher program version 4.1.4 (Genecodes, Ann Arbor, MI). The edited sequence was aligned and compared with the DNA equivalent of the ES RNA sequence using the Clustal X program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX).

4.3 Results

4.3.1 RT-LDA – optimisation and evaluation of a novel primer design for the linear type of amplification

The initial experiments for optimisation of the RT-LDA reaction were performed using long incubation times and a two-step temperature profile with reverse transcription at 42ºC for 1 hour and amplification (strand displacement/polymerization) at 53ºC for 2 hours (3 hours of RT-LDA in total). Template nucleic acid (i.e. ES RNA) was used at this stage of optimization only at high concentrations: ~ $4x10^7$ and $4x10^8$ copies/ml. Five forward primers (Fw#1, Fw#1A, Fw#1B, FW#2 and Fw#3) combined individually with the reverse primer (Rev#1) were first evaluated for the linear type of RT-LDA. Three out of five forward primers (Fw#1, Fw1A, Fw#1B) had the same HIV-1 specific sequence, but different lengths of the 5' overhang. Forward primer Fw#2 is positioned more internally to Fw#1 and has the shortest "loop" overhang" with ~ 50% GC content. Primer Fw#3 is positioned between Fw#1 and Fw#2 and partially overlaps with primer Fw#2. Each of these primers was combined individually with the reverse primer Rev#1 in separate RT-LDA reactions. Initial RT-LDA experiments revealed no amplification product for all five primer combinations using a 1% MetaPhor® gel and FRET probes for detection.

In order to exclude failure of amplification due to non-optimal RT-LDA conditions rather than a non-optimal primer design, the individual steps of the amplification reaction were assessed. To troubleshoot the RT step, ES RNA was reverse transcribed with the Rev#1 primer using different concentrations of AMV enzyme. RT was performed for 1 hour at 42ºC and 20 µl of undiluted RT reaction (cDNA) was than amplified by conventional PCR (see 4.2.2) using primers SKT145 and Rev#1. PCR product was confirmed using 1% MetaPhor® gel electrophoresis. Troubleshooting of the RT step revealed that 2.5 units of AMV enzyme used initially were insufficient and an optimal concentration of 7.5 units of AMV per reaction was established. Next, cDNA reverse transcribed with primer Rev#1 under optimised RT conditions was PCR amplified using a set of primers SKT145/Rev#1. PCR product was incubated with *Bso*BI restriction enzyme at 53ºC for 3 hours. Digested and undigested PCR products were verified using 2% MetaPhor® gel electrophoresis (Figure 4.8). The presence of the digested product confirmed functional activity of *Bso*BI at 53ºC and the presence of a corresponding restriction site in the reverse primer Rev#1.

Following this troubleshooting and optimisation of the RT step, RT-LDA reactions were performed using the combination of five forward primers described above and the Rev#1 primer. Similarly to the first round of experiments no RT-LDA amplification product was detected for all primer combinations using MetaPhor® gel and FRET hybridization probes. Next, PCR was employed to confirm the presence/absence of RT-LDA product and to exclude insufficient sensitivity of the detection formats used. RT-LDA reactions were diluted 1:50 and 1:100 in molecular grade water and 15 µl of the diluted product was used for PCR as described in section 4.2.2. PCR was performed with the same primer combination that was originally used for each RT-LDA reaction (e.g. Fw#2/Rev#1, Fw#1A/Rev#1, etc.).

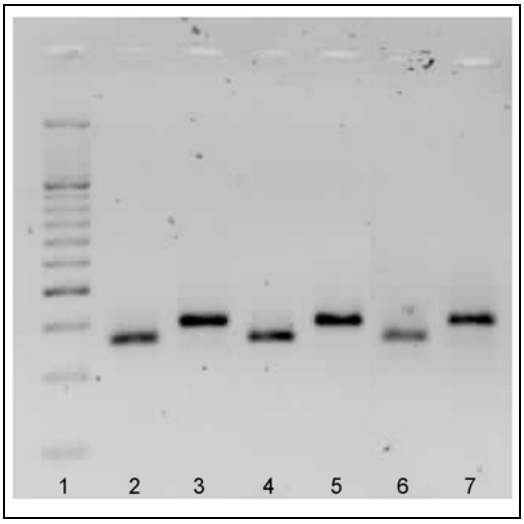


Figure 4.8: MetaPhor® gel (2%) showing PCR products digested with BsoBI restriction. enzyme. Lane 1 – DNA ladder (50 bp); lanes 2, 3 – PCR product amplified from ES DNA (positive control), digested product (2) and undigested (3); lanes 4, 6 - digested PCR products amplified from the different cDNA templates; lanes 5, 7 – the corresponding undigested PCR products.

For each primer combination the DNA equivalent of ES RNA (i.e. purified dsDNA amplification product obtained using ES RNA and a conventional RT-PCR) was used as a positive control and a corresponding RT-LDA no template control (NTC) sample was use as a negative control. PCR revealed the presence of RT-LDA product only in the reaction performed with the set of primers Fw#2 and Rev#1. Together with a specific amplification product the RT-LDA reaction and the blank sample revealed the presence of a non-specific product (data not shown). Since the non-specific product was found in a blank RT-LDA sample as well as in RT-LDA reaction it is likely to be due to the presence of partially complementary regions in primers Fw#2 and Rev#1 causing formation of this product under the low stringency isothermal amplification conditions. The specific RT-LDA product further amplified by PCR was excised from the gel, purified and used for

sequencing with a set of primers Fw#2 and Rev#1 (section 4.2.3). The nucleotide sequence of PCR amplified RT-LDA aligned with the ES DNA is shown in Figure 4.9. These experiments demonstrated successful RT-LDA using a pair of primers Fw#2 and Rev#1. However, due to the presence of a non-specific amplification product the primer design required further improvement.

In order to prevent formation of a non-specific product another two forward primers (Fw#3A and Fw#1C) were designed and evaluated in combination with the primer Rev#1. RT-LDA reactions were performed using a two step temperature profile and long amplification time as described above, and two combinations of primers: Fw#1C/Rev#1 and Fw#3A/Rev#1. FRET hybridization probes revealed RT-LDA product amplified using both newly designed primers (Fw#1C and Fw#3A), but the fluorescent signals were weak (data not shown).

The presence of RT-LDA product was confirmed with conventional PCR. RT-LDA samples amplified with primers Fw#1C/Rev#1and Fw#3A/Rev#1 and their corresponding blank samples were diluted 1:50 and 15 µl of each diluted sample was further PCR amplified with the corresponding RT-LDA primers. ES DNA was used as a positive PCR control for both combinations of primers. PCR product was detected in RT-LDA samples and in two positive control samples, but not in the RT-LDA blank samples (Figure 4.10). Non-specific product observed using initially designed primers was not present in any of these PCR samples. Significantly less amount of PCR product was generated using the set of primers Fw#1C/Rev#1 than using the set of primers Fw#3A/Rev#1, which reflects lower efficiency of RT-LDA reactions performed with primers Fw#1C/Rev#1 (Figure 4.10).

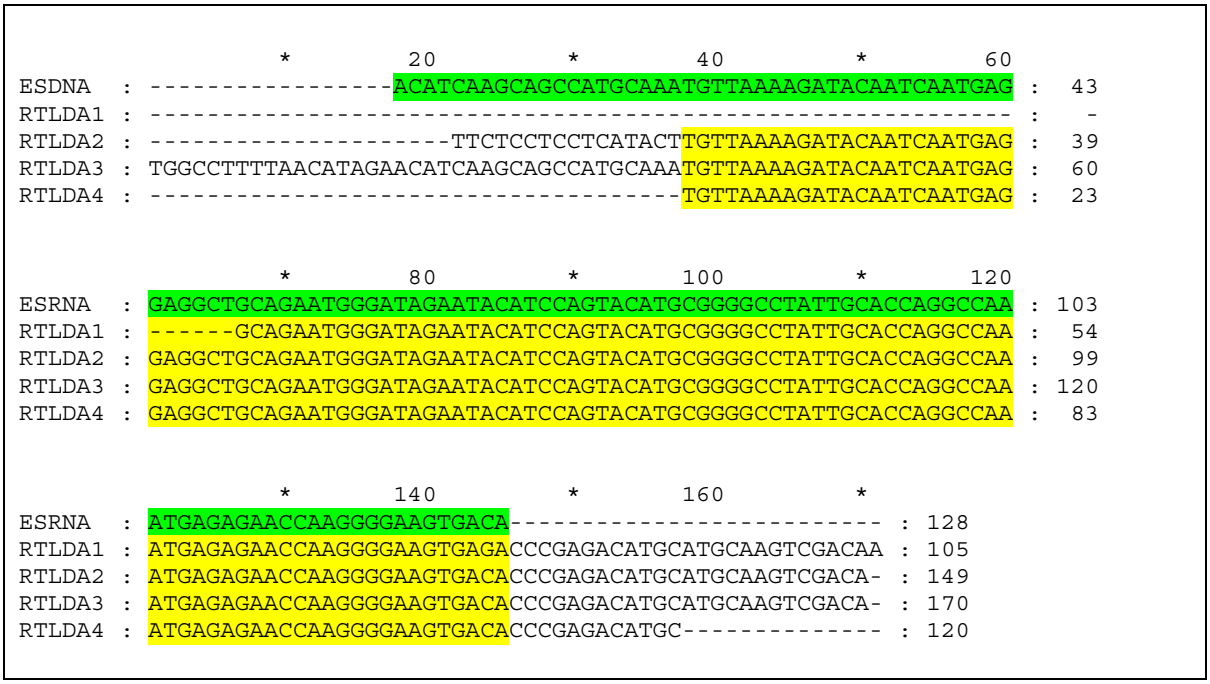


Figure 4.9: Sequence alignments. Nucleotide sequences obtained from four RT-LDA reactions performed using 4 different combinations of primers are aligned with the ES DNA sequence (i.e. DNA equivalent of ES RNA sequence; the ES RNA transcript that was used as a template in the RT-LDA reactions). The sequence of ES DNA (128 bp) is highlighted in green; test amplicon sequences (excluding primer binding sites) are highlighted in yellow. RT-LDA product n#1 (~ 105 bp) was amplified and sequenced using primers Fw#2/Rev#1; RT-LDA product n#2 - Fw#3A/Rev#1 (~ 149 bp); RT-LDA product n#3 - Fw#1C/Rev#1 (~ 170 bp); RT- LDA product n#4 was PCR amplified and sequenced using the primer set Fw#3A/Rev#1 (~ 120 bp). (Note: for the RT-LDA product n #4 primers Fw#3A was used instead of Fw#4a since it has a much shorter overhang to provide more optimal PCR and sequencing reactions).

PCR products obtained with both sets of primers were gel purified and sequenced (see section 4.2.3). Sequencing revealed the presence of HIV-1 specific sequence in PCR products amplified using both sets of primers (Figure 4.9).

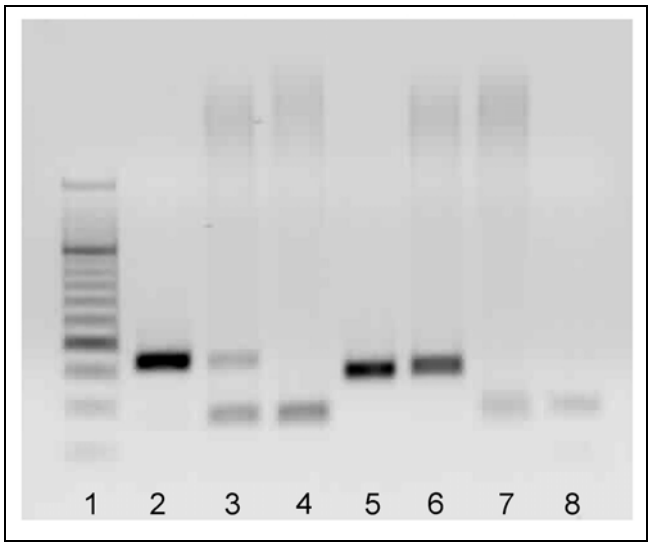


Figure 4.10: MetaPhor® gel (1%) showing PCR product amplified from the linear RT-LDA reactions. Lane1- DNA ladder (50 bp); lane 2- ES DNA directly amplified by PCR using primers Fw#1C/Rev#1 (~ 170 bp; positive control for this primer set); lane 3 – PCR product amplified from RT-LDA using primers Fw#1C/Rev#1 (~ 170 bp specific product and ~ 90 bp non-specific product); lane 4 – corresponding RT-LDA blank sample containing only a non-specific product; lane 5 – ES DNA control directly amplified with primers Fw#3A/Rev#1 (~ 150 bp); lane 6 - PCR product amplified from RT-LDA using primers Fw#3A/Rev#1 (~ 150 bp); lane 7 – corresponding RT-LDA blank sample amplified by PCR (no product); lane 8 – PCR blank sample (no template control - NTC) showing no amplification product.

4.3.2 RT-LDA – optimisation and evaluation of a novel primer design for the semi-cycling type of amplification

The next phase in the development process involved a further modification that was applied to LMP forward primers and converted RT-LDA into a semi-cycling amplification method (Figure 4.7). Based on the results of preliminary optimisation and evaluation of the linear type of RT-LDA, the forward primer Fw#3A had the most optimal design. Thus, primer design for a semi-cycling RT-LDA incorporated the features of primer Fw#3A. In particular, primer Fw#4A includes the same HIV-1 specific sequence and the loop

overhang sequence as primer Fw#3A and in addition it has a nickable site for *Bso*BI and the random primer sequence (Figure 4.7). New forward primer Fw#4A designed for semi-cycling RT-LDA was evaluated in combination with primer Rev#1 using high concentrations of ES RNA ~ $4x10^7$ and $4x10^8$ copies/ml and reaction conditions as described in section 4.3.1. Detection with FRET probes demonstrated the presence of amplification product in RT-LDA reactions performed with primer sets Fw#4A/Rev#1 and no product in the blank sample (Figure 4.11). The characteristic melting temperature (T_m) observed for RT-LDA amplicons using these FRET probes was ~ 68.8-71ºC. Unambiguous detection of RT-LDA amplicons with FRET probes was achieved and allowed further optimisation of this novel method. Thus, for optimization and preliminary evaluation of semi-cycling RT-LDA reactions only the post-amplification melting curve analysis with FRET hybridization probes was used for detection of product. RT-LDA reaction components such as DMSO and phosphate buffer affect the specificity and reproducibility of amplification. The concentration of DMSO was titrated from 4 to10% v/v and the concentration of phosphate buffer from 35mM to 50 mM. It was found that an increased concentration of DMSO requires higher concentrations of phosphate buffer to maintain sufficient amplification. Preliminary data obtained from the NALF/MAR™ feasibility study conducted by BBI demonstrated that higher concentrations of phosphate buffer decrease the intensity of magnetic signal detected with MAR™. Thus, high (8 - 10%) concentrations of DMSO that provide greater specificity of primer annealing required higher concentrations of phosphate buffer, which interferes with the MAR™ detection.

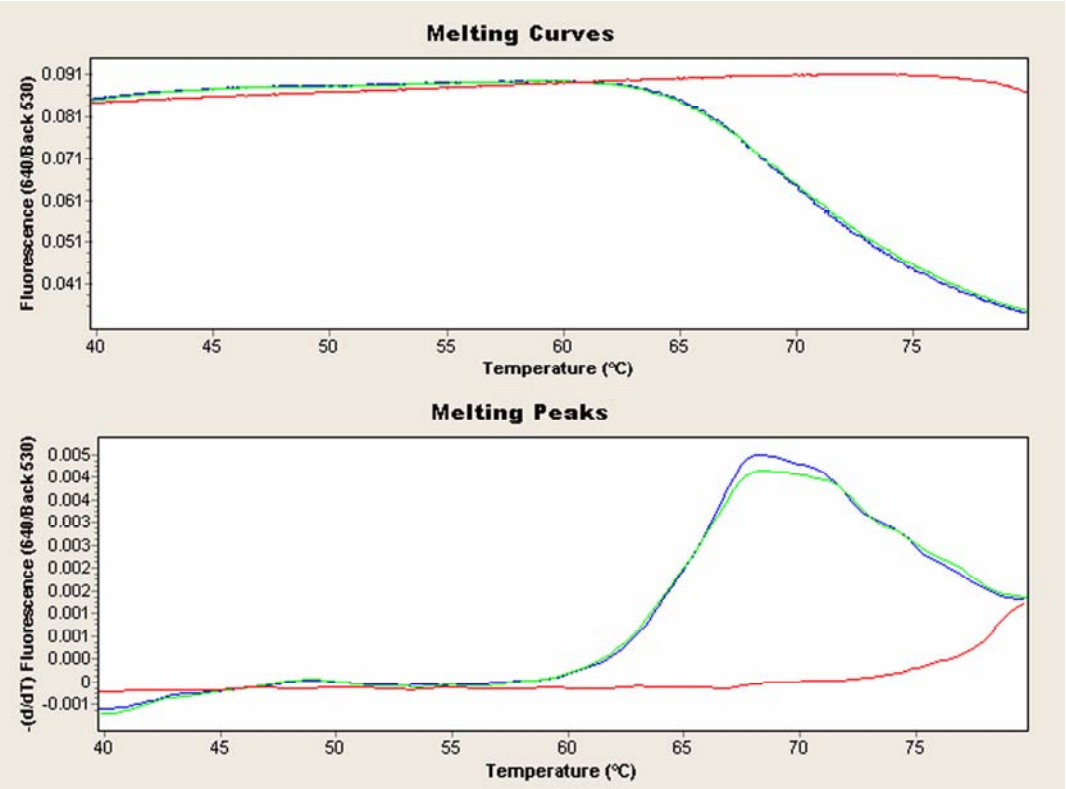


Figure 4.11: Melting curve analysis using FRET probes for detection of RT-LDA product. Melting curve profiles of both RT-LDA samples (blue and green lines) show the presence of the specific amplification product with a typical melting peak at ~ 69°C. No melting peak is observed in the RT-LDA blank sample (red line).

Taking into account the desire for compatibility of RT-LDA with the MAR™ detection platform, the most optimal concentrations of both components were established at 35 mM for phosphate buffer and 5% for DMSO. Next, the concentrations of primers Fw#4A and Rev#1 were titrated in the range of 0.5 µM, 0.75 µM and 1 µM. Each concentration of primers was tested in duplicate using two ES RNA concentrations of $4x10^7$ and $4x10^9$ copies/ml. A concentration of primers at 0.75 µM was found to be the most optimal for both concentrations of template ES RNA tested (data not shown). Further optimization of the RT-LDA aimed to reduce the total amplification time (< 3 hours) and to perform the amplification at only one constant temperature (53ºC). The initial experiment for time reduction was performed using two –step temperature profile as follows: RT step at 42ºC

for 30 min and amplification step at 53ºC for 1 hour. Total reaction time was halved from 3 hours to 1.5 hours using this approach. For this experiment a wide range of ES RNA concentrations was used: $4x10^3$, $4x10^4$, $4x10^5$, $4x10^6$, $4x10^7$, $4x10^8$ copies/ml. RT-LDA amplicons were detected in all six samples using FRET probes and the melting curve analysis on the LightCycler® platform (Figure 4.12). Based on this successful experiment, further reduction in amplification time was attempted using only one constant temperature for both the RT and amplification steps. Two amplification profiles were tested for the RT-LDA reactions: one at 53ºC with a total reaction time of 30 minutes and another profile was performed at 53ºC for 1 hour. Both amplification profiles were performed using a range of ES RNA concentrations: of $4x10^3$, $4x10^4$, $4x10^5$, $4x10^6$, $4x10^7$, $4x10^8$ copies/ml. RT-LDA product was detected using FRET probes in all samples (excluding a blank sample) amplified for 30 minutes and 1 hour respectively. Three RT-LDA samples were used for PCR to provide an additional confirmation of successful RT-LDA using the new times and temperature profiles. In particular, the RT-LDA samples containing ES RNA at a starting concentration of $4x10^7$ and $4x10^6$ copies/ml that were amplified for 1 hour and a sample containing $4x10^3$ copies/ml of ES RNA amplified for 30 minutes were diluted 1:10 and further amplified by PCR using primers Fw4A/Rev#1. PCR product for the RT-LDA sample of $4x10^7$ copies/ml was clearly detected as a bright band on the 1% MetaPhor® gel; the other two samples revealed faint bands (data not shown).

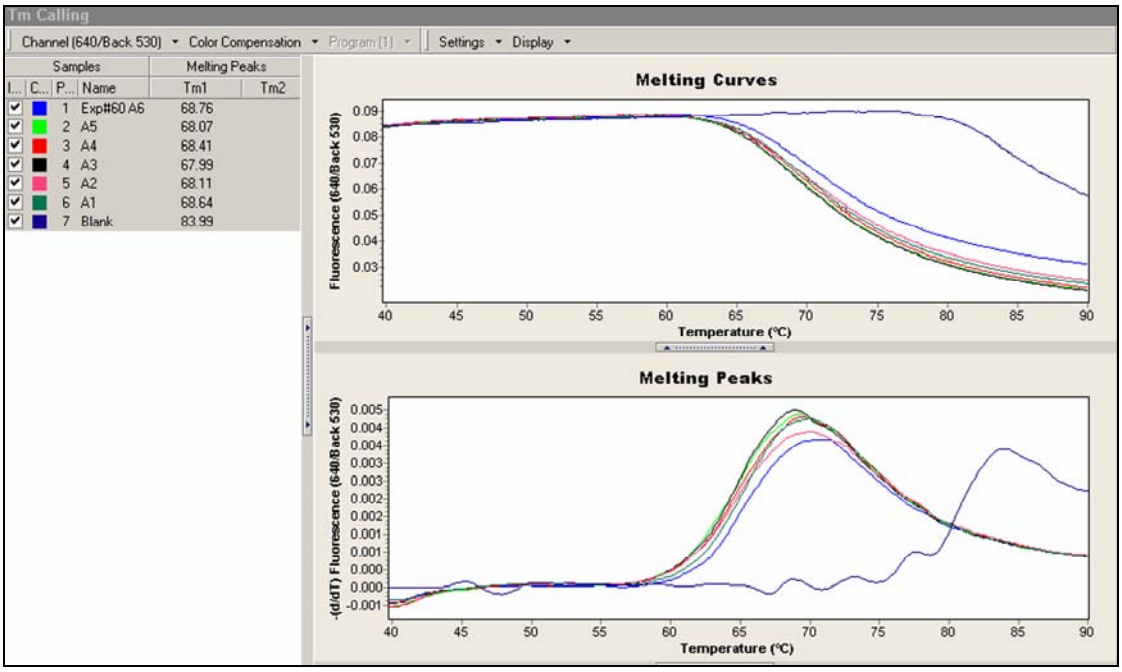


Figure 4.12: Detection of RT-LDA product using FRET probes. After 90 minutes of RT-LDA melting peaks (~ Tm at 68-69ºC) are observed in all samples from A1 to A6 with the corresponding concentrations: $4x10^3$, $4x10^4$, $4x10^5$, $4x10^6$, $4x10^7$, $4x10^8$ ES RNA copies/ml. These melting curve profiles reflect the presence of an HIV-specific amplification product detected with the FRET probes. The blank sample (blue line) shows a peak at ~ 84ºC, which is either due to the presence of a non-specific product or represents a detection artefact. This type of high melting peak was non-reproducible between the experiments.

In order to confirm the presence of the specific product in these faint bands 18 µl of both ($4x10^3$ and $4x10^6$ copies/ml) PCR reactions and 18 µl of the PCR product of the ES DNA control were used for FRET detection on the LightCycler®. The specific PCR product was detected using the melting curve analysis in all samples except the blank sample (Figure 4.13).

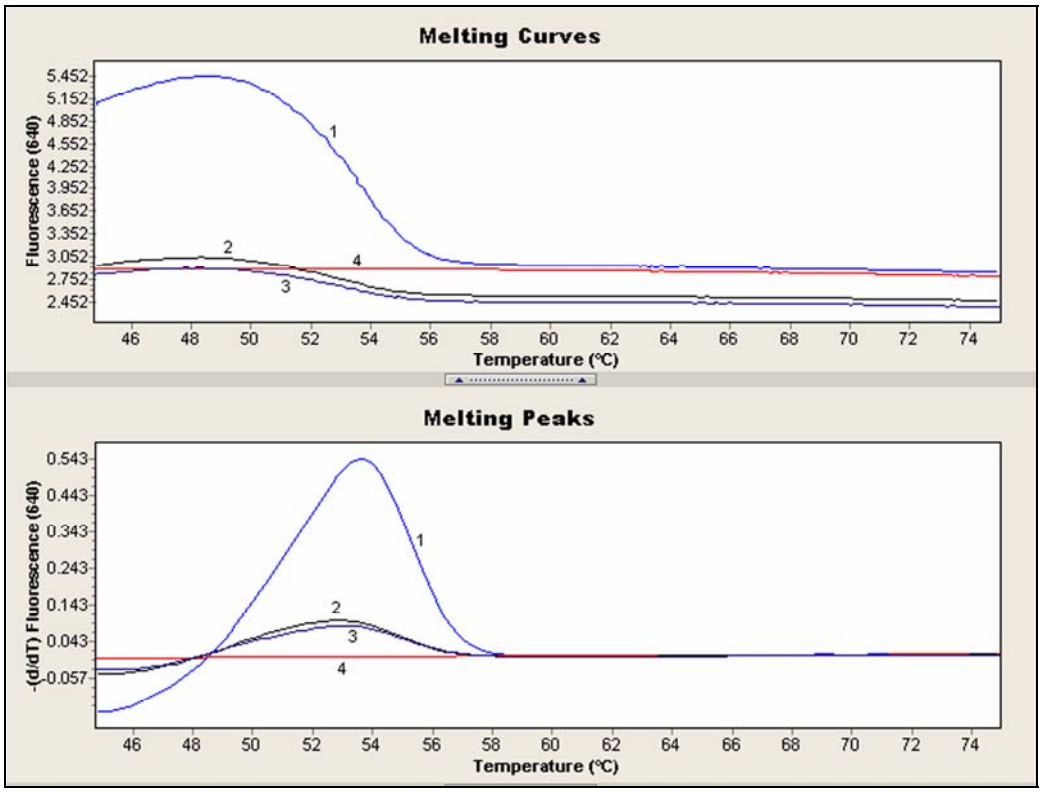


Figure 4.13: Melting curve analysis using FRET probes for verification of PCR product. Melting peaks at ~ 53ºC indicate the presence of a specific amplification product in the samples: 1 – ES DNA control; 2 and 3 - RT –LDA samples with input template concentrations of $4x10^3$ and $4x10^6$ ES RNA copies/ml, respectively; 4 – PCR blank sample shows no detection of product. (Note: The temperature of melting peaks for PCR products is lower (~53ºC) than for RT-LDA products (68-69ºC) even though the same FRET probes are used for detection. This is due to different reaction mixes and salt compositions used in each type of amplification.

RT-LDA product obtained with high ES RNA concentrations ($4x10^7$, $4x10^8$ copies/ml) and primers Fw4A/Rev#1 was amplified again using conventional PCR and primers Fw3A/Rev#1. Primer Fw#3A has the same HIV-1 sequence specific region as the primer Fw#4A and was used due to the limited amounts of the primer Fw#4A available. PCR product (~ 150 bp) was gel purified and used for sequencing (Figure 4.14). The sequence obtained using primers Fw#4A and Rev#1 was aligned with the ES DNA (Figure 4.9).

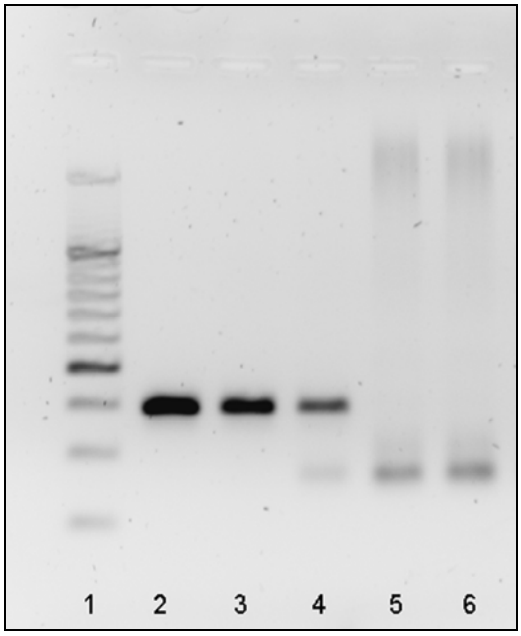
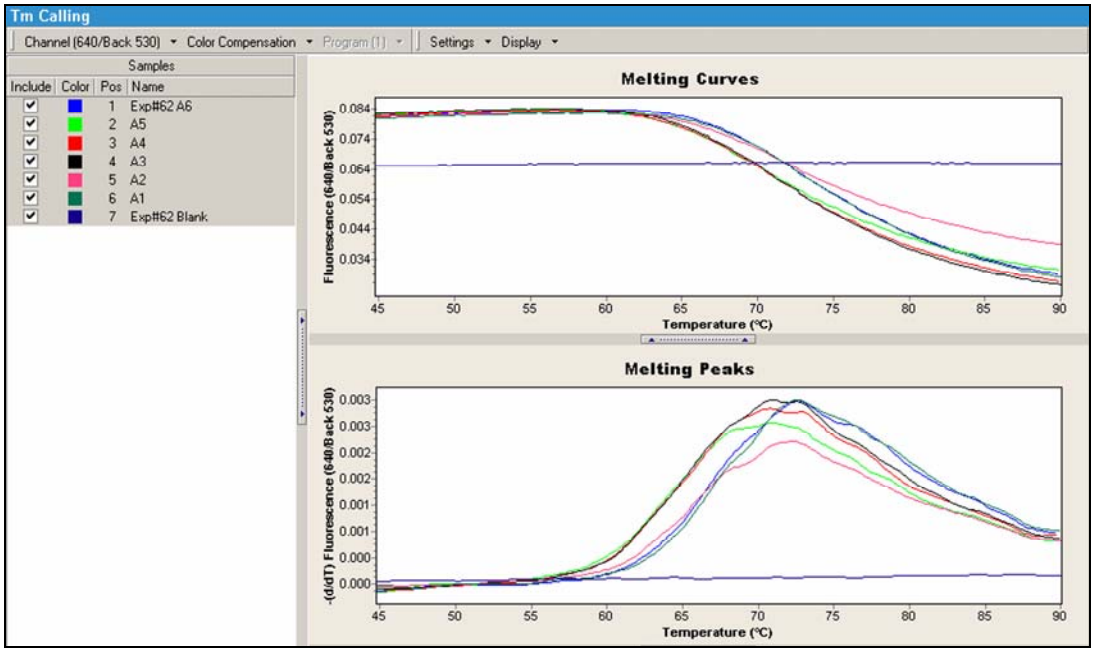


Figure 4.14: MetaPhor® gel (1%) showing PCR product amplified from the semi-cycling RT-LDA reactions. Lane 1 – DNA ladder (50 bp); lane 2 – ES DNA used as a positive control for PCR shows the presence of a specific product (~ 150 bp); lanes 3 and 4 - PCR product obtained from further amplification of RT-LDA product (ES RNA input in the RT-LDA reaction was $4x10^8$ and $4x10^7$ copies/ml respectively); lanes 5 and 6 – NTC (no template control) samples for RT-LDA and PCR, respectively shows the presence of primer dimers only.

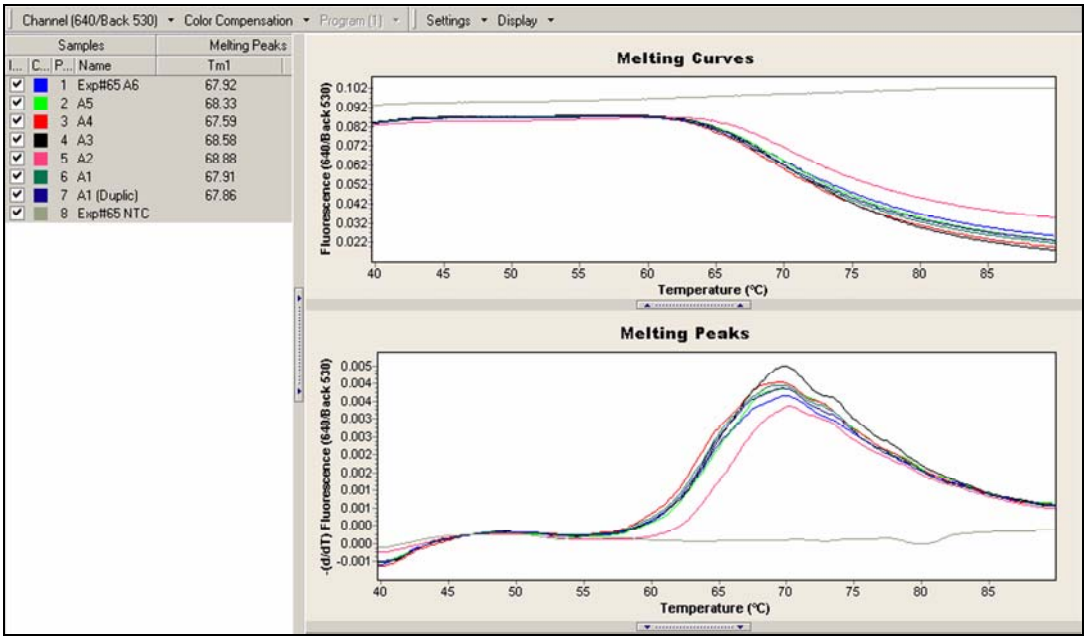
Since successful RT-LDA was demonstrated using 1 hour and even 30 minutes amplification time at 53ºC, initial evaluation of the method was performed to assess reproducibility of semi-cycling RT-LDA under the new conditions. Reproducibility of RT-LDA was studied in five different experiments performed on five different days. Each experiment was performed using serial dilutions of a template ES RNA covering a range of concentrations: $4x10^3$, $4x10^4$, $4x10^5$, $4x10^6$, $4x10^7$ and $4x10^8$ copies/ml. RT-LDA reactions were performed at 53ºC for 1 hour. Post-amplification detection of amplicons was performed using melting curve analysis and FRET hybridization probes. RT-LDA product was detected using FRET probes in all samples in each of the five repeated runs. Thus, no

failed amplification was observed in any of the RT-LDA reactions performed on five different days using a concentration range of ES RNA covering six orders of magnitude. Figure 4.15 shows melting curve analysis for two out of five repeated RT-LDA experiments.

Preliminary evaluation of semi-cycling RT-LDA also involved detection of the amplicons using anti-DNP striped dipsticks and NALF. RT-LDA reactions were performed using the short amplification profile described in section 4.2.1.2 and ES RNA template at concentrations of $4x10^8$ and $4x10^7$ copies/ml. Different dilutions (from 1:10 to 1:30) and input concentrations (from 1 µl to 30 µl) of the diluted RT-LDA product were used for NALF detection. The samples that gave positive detection in a visual detection range of NALF are shown in Figure 4.16. The most optimal detection was found in samples diluted 1:10 and 10-30 µl of that dilution. Reproducibility of NALF detection of the RT-LDA amplicons was confirmed in a number of lateral flow experiments.



A



B

Figure 4.15: Melting curve analysis for repeated RT-LDA experiments.

RT-LDA reproducibility experiments (2 out of 5) are illustrated in figure A and B, respectively. Specific RT-LDA amplification product is detected using FRET probes and melting curve analysis on the LightCycler® platform (Tm ~ 68.8 -71ºC) for the ES RNA concentration range of 4x103-4x10^8 copies/ml. No amplification product is detected in NTC samples.

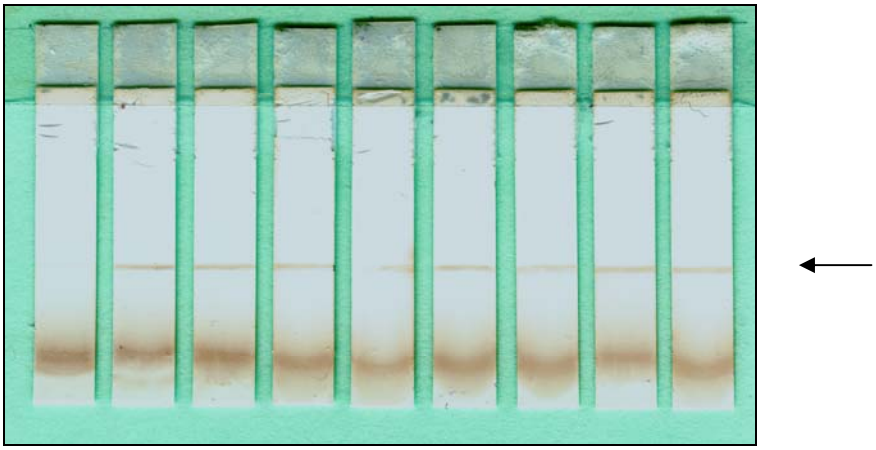


Figure 4.16: Detection of RT-LDA amplicons (arrow) using NALF dipsticks.
From left to right: RT-LDA blank sample (1); two positive controls containing an artificially synthesized looped amplicon (ampi29) (2, 3); the next 6 dipsticks show the detection of diluted RT-LDA amplification products obtained from the different experiments (4-9).

4.4 Discussion

Nucleic acid testing for diagnosis and monitoring of infectious diseases offers certain advantages, which makes it an attractive alternative for adaptation in rapid POC diagnostics. In particular, NAT provides significantly higher analytical sensitivity and accuracy of quantitation when compared to serologic or antigen-based assays. Nucleic acid amplification based assays usually comprise of three main steps: isolation of nucleic acids, target or/and signal amplification and detection of amplification product. Recently there has been an increasing interest in developing miniaturised instruments containing inexpensive, disposable test units (e.g. microfluidic cassettes) that allow performing amplification and detection steps of NAT in one closed system suitable for the near-patient

testing (Wang et al., 2006). Nucleic acid lateral flow represents a low cost, rapid detection system widely used for POC testing. Visual or instrument dependant detection of product on the NALF strips is achieved by using different reporter particles, for example, dye-encapsulating liposomes - biosensors (Edwards and Baeumner, 2006; Nugen et al., 2006). Dineva and colleagues (Dineva et al., 2005) report on the development of simultaneous visual detection of amplicons from HBV, HCV and HIV-1 using lateral flow dipsticks and antibodies conjugated to the colloidal gold particles. This inexpensive, rapid and instrument independent detection format showed good sensitivity allowing detection of 50, 125 and 500 IU/ml for HBV DNA, HCV RNA and HIV-1 RNA respectively. Multiplex amplification of all three pathogens was performed using conventional RT-PCR. Although in its current format this assay (Dineva et al., 2005) represents a laboratory based assay, the study demonstrates a potential for adapting NAT for POC diagnostics.

The feasibility study described in this chapter reports on the development of a novel method for isothermal amplification of RNA, termed RT-LDA. RT-LDA is designed to fulfil the criteria required for POC diagnostics: rapid and efficient amplification of ssDNA amplicons using viral RNA template. The RT-LDA technique combines an innovative primer design (LMP) and a known concept of strand displacement amplification using restriction enzyme *Bso*BI and *Bst* DNA polymerase. The RT-LDA reverse primer, similarly to primers used for strand displacement amplification, possesses a *Bso*BI recognition site that gets nicked and this ssDNA nick initiates a series of strand displacement/polymerisation driven by *Bst* polymerase (Hellyer et al., 1999; Nadeau et al., 1999; Nycz et al., 1998; Spargo et al., 1996; Walker, 1993; Walker et al., 1995; Walker et al., 1992; Walker et al., 1996; Walker et al., 1994). The LMP design was applied to the RT-LDA forward primer - it possesses a 5' overhang sequence, which is complementary to

the sequence of a sense strand extended from this primer. During the RT-LDA reaction dsDNA targets (T1, T2 and T3) are formed (Figure 4.7), and subsequent displacement of multiple anti-sense strands/amplicons begins. During strand polymerisation these strands/amplicons copy an overhang from the forward primer. Thus, the displaced anti-sense amplicons have two regions at their 5' end with complementary sequences, which hybridize to each other causing the partially "looped" structure of the ssDNA amplicons (Figure 4.5). Accumulation of partially "looped" ssDNA amplicons represents a key feature of RT-LDA. The "loop" structure closes the region complementary to the forward primer and thus, prevents conversion of anti-sense ssDNA amplicons into dsDNA amplicons. Another novelty applied to RT-LDA design is the absence of bumping primers that are normally used in the SDA technique. This is done to avoid production of multiple dsDNA species seen in SDA (Figure 4.4) and make RT-LDA less complex. Instead of bumping primers, RT-LDA makes use of AMV reverse transcriptase that has combined RNase H activity, which digests viral RNA in the RNA/cDNA hybrid leaving cDNA in a single stranded form. The functionality of the novel modifications applied to the RT-LDA design were proven experimentally.

Two variations of the RT-LDA technique were designed and evaluated: linear and semi-cycling (Figures 4.6 and 4.7). Originally the RT-LDA method with a linear type of amplification was designed. Semi-cycling RT-LDA was designed at the later stage, after preliminary evaluation of linear RT-LDA. Initial experiments were performed to set up a novel technique and find the most optimal design for a RT-LDA forward primer containing the "loop" overhangs. Theoretically, the "loop" overhang has to be complementary to the short sequence at the 3'end of the forward primer and to the longer sequence of the extended sense strand. The length of a complementary "loop" sequence has to be long

enough to preserve a single stranded structure of DNA amplicons, but not too long to interfere with the extension of a forward primer. The exact dimensions of the "loop" overhang in terms of the number of nucleotides and GC content were established experimentally. This was achieved by performing RT-LDA reactions using 7 forward primers with the 5'- "loop" overhangs of different length and sequence composition. According to the experimental data obtained for linear RT-LDA reactions, the most optimal design for RT-LDA forward primer suggests short sequence (9 bases) of a "loop" forming overhang with a high (~50%) GC% content. However, the amplicons produced with the linear RT-LDA method could not be sufficiently detected using FRET hybridization probes and the LightCycler® platform. RT-LDA product from linear amplification could only be seen by further amplification using conventional PCR and was confirmed additionally using DNA sequencing. This shortfall was attributed to the insufficient power of amplification provided by the linear RT-LDA.

The new design for semi-cycling RT-LDA aimed to improve the magnitude of amplification of the technique making it more suitable for POC testing. The modification was achieved by the addition of the second *Bso*BI nickable site into the sequence of the forward loop mediating primer (Figure 4.7 A). This modification of the LMP does not interfere with displacement of ssDNA partially "looped" anti-sense amplicons. The modified forward LMP enhances amplification by at least 3- fold due to formation of three different types of dsDNA targets (T1, T2 andT3) as opposed to only one such target in the linear type of RT-LDA design (Figures 4.5 and 4.6). In particular, modification was applied to a forward primer Fw#3A, which was shown previously to produce the most optimal linear amplification. For ease of differentiating between primers a modified Fw#3A containing a *Bso*BI restriction site and a random sequence for "an anchoring

artificial" primer was termed Fw#4A (section 4.2.1.1). RT-LDA experiments performed with new primer Fw#4A and Rev#1 immediately showed an improved detection using melting curve analysis with the FRET hybridization probes. Optimisation of reaction conditions allowed further improvement of RT-LDA. Reduction in amplification time of RT-LDA that was performed using one constant temperature (53ºC) revealed good sensitivity of this method. In particular 4000 copies/ml of ES RNA template could be detected within only 30 minutes amplification. PCR product amplified from RT-LDA using a primer set Fw#4A/Rev#1, confirmed the presence of a specific product only. Sequence identity of each PCR product was confirmed by aligning the amplicon sequences with HIV-1 subtype C sequence (Figure 4.9). Preliminary evaluation of semi-cycling RT-LDA was performed using a wide range of ES RNA concentrations from $4x10^3$ to $4x10^8$ copies/ml, which were tested in five replicate RT-LDA experiments. Each RT-LDA run was performed at 53ºC for 1 hour. Repeated experiments showed that the newly designed RT-LDA technique is reproducible over six orders of magnitude. Preliminarily, the sensitivity of the one hour RT-LDA reaction combined with the FRET detection format can be set at 4000 copies/ml. According to the design of POC HIV-1 RNA test (Figure 4.1) NALF in combination with the MAR™ instrument represent a final detection format. Preliminary evaluation of RT-LDA also included NALF experiments in order to demonstrate compatibility of this new amplification technique with NALF detection. It was important to exclude possible interference between NALF reaction components and RT-LDA reactions mix. Most importantly the detection of RT-LDA product using NALF dipsticks, without a prior denaturation step, provides experimental proof of the single stranded structure of the partially looped RT-LDA amplicons. Since the MAR™ instrument was not available in our setting during the initial assay development, NALF detection was performed in a visual range. Positive detection observed for RT-LDA

reactions showing that the newly developed amplification technique is fully compatible with NALF detection using anti-DNP striped dipsticks and anti-superparamagnetic conjugates.

Overall, the newly developed isothermal amplification method – RT-LDA, offers a unique feature of converting RNA templates into ssDNA amplicons. A number of applications require ssDNA product of a defined length for further manipulation, for example, detection of nucleic acids by flow cytometry (Horejsh et al., 2005). Protocols have been developed that describe time consuming and cumbersome post-amplification treatment of dsDNA amplicons in order to convert them into ssDNA amplicons (Kuo, 2005; Nagamine et al., 2002). The RT-LDA method generates ssDNA product and can be directly combined with detection systems like NALF, flow cytometry and microarrays. Further modifications can be made to the reaction conditions of RT-LDA to improve and simplify detection. For example, instead of using a capture and a detection probe format, single probe detection for NALF (Piepenburg et al., 2006) can be applied to RT-LDA. Addition of NALF probe at the beginning of RT-LDA may further reduce overall assay time.

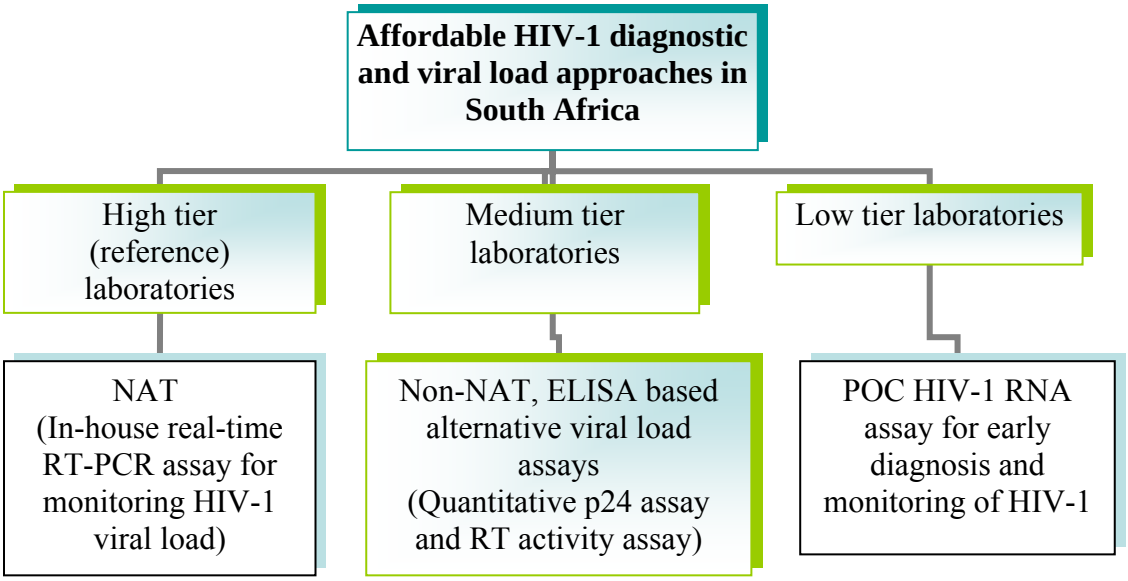
In conclusion, preliminary evaluation demonstrated that the novel RT-LDA technique (semi-cycling version) is reproducible over a wide range of template concentrations ($4x10^3$ - $4x10^8$ copies/ml) and provides good sensitivity using only one hour of amplification. RT-LDA is fully compatible with NALF detection using dipsticks and thus, the new method can be used as an amplification front end for a novel POC HIV-1 RNA diagnostic test. It is envisaged that future optimisation and evaluation of RT-LDA using the MAR™ instrument and clinical specimens will allow for the implementation of this technology in a clinical setting, e.g. at VCT clinics, to detect acute HIV-1 infection.

CHAPTER 5

General discussion and future development

This thesis describes innovative approaches to developing more affordable HIV-1 diagnostic and monitoring assays for resource limited settings (see diagram below). The study design takes into account the available laboratory infrastructure in South Africa represented by high tier, reference laboratories (~ 21%), medium tier laboratories (~ 10.2%) and low tier laboratories at the primary health care clinics (68.8%). This type of infrastructure with different levels of laboratories unevenly distributed throughout the country eliminates a single solution for all laboratories. This mirrors the situation in numerous laboratory environments in other developing countries. Development of less expensive in-house assays as well as evaluation of more affordable commercial alternatives for monitoring HIV-1 viral load will facilitate the implementation of ARV treatment programs in South Africa (see Chapter 1).

Commercial NAT for monitoring HIV-1 viral load is currently used in high volume laboratories in tertiary settings in South Africa. These assays include the NucliSens EasyQ (BioMérieux) and the COBAS Amplicor (Roche). The assays have good clinical performance, but are expensive to implement and maintain in a developing country environment with limited resources. Moreover, none of these assays provides sufficiently high throughput for the laboratories that need to process several hundred samples per day. Development of a less expensive in-house assay that has a potential for higher throughput will address this issue and was thus investigated in this project.



The successful development and preliminary clinical evaluation of an in-house viral load assay – the LUX assay is described in Chapter 2. The LUX assay makes use of real-time PCR technology and a LUX™ primer as a detection format. Real-time PCR technology offers a combined advantage of quick turnaround time, high throughput and performance of amplification and detection of PCR product in a closed system thus reducing contamination, an extremely valuable feature for a diagnostics laboratory. The LightCycler® is one of the most widely used real-time PCR platforms and it was selected based on its advanced technical characteristics and due to the availability of the Roche Diagnostics and Applied Sciences agency in South Africa. The agency provides a sufficient number of trained engineers and molecular research consultants who provide adequate support. All necessary reagents, consumables and technical assistance are readily available from the company within 1 day to a maximum of 10 working days. PCR reagents used in the LUX assay are obtained from Qiagen through the local Southern Cross Biotechnology agency. Qiagen is one of the leading international companies in manufacturing reagents for molecular research. Similarly to the Roche agency, technical assistance and reagents are available from the Southern Cross Biotechnology agency

within 1 – 14 working days. Supply of reagents and availability of the technical support are essential for successful implementation of a new technology in a diagnostics laboratory. Good clinical performance of the LUX assay was demonstrated using South African HIV-1 subtype C stored specimens. The assay shows good specificity and analytical sensitivity of 400 RNA copies/ml. The LUX assay shows good agreement with the COBAS Amplicor assay. Evaluation of the LUX assay on a longitudinal patient cohort on HAART showed that the newly developed assay reflects the expected decline in HIV-1 viral load over time. Viral rebounds in some patients were simultaneously detected by both the LUX and the COBAS Amplicor assays. Thus, the LUX assay represents a potential, more affordable alternative for high volume viral load monitoring in South Africa. Preliminary cost estimation shows that the LUX assay (cost of reagents and consumables) is 2-3 times less expensive than the NucliSens EasyQ and the COBAS Amplicor assays. Negotiations with the suppliers have been initiated and a further 10% discount on RT-PCR reagents is quite feasible. Future development of the LUX assay will aim to further reduce the cost of the assay. It is also envisaged to transfer the assay onto the latest, high throughput version of the LightCycler® system (version 480) and evaluate the LUX assay using fresh plasma samples. The LightCycler® 480 offers a significantly higher throughput (96 to 384 samples per run) than the most current commercial assay (Table A1, Appendix A). Although the new real-time PCR system is expensive we hope that the large number of tests will warrant discounts on the reagents and consumables used for the LUX assay.

More affordable commercial alternatives for viral load monitoring were also evaluated in this study (Chapter 3). The HiSens™ HIV-1 p24 Ag Ultra assay (Perkin-Elmer) and the ExaVir® Load kit (Cavidi) for reverse transcriptase (RT) enzyme activity assay are non-NAT, ELISA based assays, which represent a less complex option for implementation in

medium tier laboratories with a simplified set-up. The p24 antigen and the RT activity assays are significantly less expensive than the RNA based assays (NAT) with an approximate cost per test of $10 and $10- $20, respectively. Implementation of these assays and equipment maintenance is also less expensive when compared to NAT. Minimal training is sufficient to perform the assays. Technical assistance and training are available in South Africa through the local agencies. Evaluation of these assays using South African HIV-1 subtype C specimens obtained from the cohort of longitudinally followed-up patients on HAART demonstrated significant association between these assays and the Roche Amplicor assay. In this cohort of patients, similarly to the Roche Amplicor assay both the p24 antigen and the RT activity assays showed a downward trend in viral load values over time in response to therapy. These results suggest that both alternative assays hold a potential for use in clinical management of patients in resource limited settings. However, insufficient sensitivity of the p24 assay and low throughput of the RT enzyme activity assay are still of a major concern. More extensive evaluation of the newer, improved versions of these assays is required, especially in subtype C, before a final decision can be made about their implementation in diagnostics.

Low tier laboratories, as mentioned above, represent the majority of NHLS laboratories in South Africa (~68.8%). These facilities are mostly located in the rural areas where patients often struggle to come back for their results on time. POC HIV-1 testing would be the most suitable approach for this type of scenario. A rapid and cost effective method for detection of viral RNA could provide a means for early diagnosis of acute HIV-1 infection in the window period. The development of a POC RNA assay is one of the most recent projects that has been initiated at WITS University and NHLS in collaboration with BBI (UK). The desired outcome for this project is to develop an instrument suitable for near-patient

testing, which could provide rapid and sensitive detection and possibly quantitation of HIV-1 RNA specific product. The POC type of instrument should ideally be easy to operate and it would allow performing main steps of the assay like RNA isolation, amplification and detection in a closed format using inexpensive (plastic or other material) disposable test cartridges and dipsticks. Possibilities of using lyophilized enzymes that can be stored at 4ºC have been provisionally discussed with BBI. Chapter 4 of this thesis describes the proof of concept study, which demonstrates the usefulness of a novel isothermal amplification method for the proposed POC HIV-1 RNA assay. The RT-LDA method was optimised and its performance characteristics were evaluated using a synthetic RNA transcript (ES RNA) containing 128 bases of HIV-1 *gag* sequence. A unique feature of RT-LDA is the ability to rapidly and efficiently convert RNA into short ssDNA amplicons under a constant temperature (53ºC). Partially looped ssDNA amplicons can be directly hybridized to the lateral flow probes without prior denaturation steps. Preliminary evaluation of the RT-LDA technique using ES RNA showed that the new method provides an efficient amplification of a wide range of template concentrations from $4x10^3$ - $4x10^8$ RNA copies/ml in one hour. RT-LDA amplicons can be detected on the dipsticks with a signal from superparamagnetic particles detectable in a visual range (with no further signal amplification). Thus, full compatibility of this method with the NALF detection format was confirmed. Since the diluted reaction mixes were used for detection on the dipsticks it may be possible to reduce RT-LDA reaction time to less than one hour. Therefore, the encouraging preliminary results warrant further development of the RT-LDA technique. Future development and evaluation of RT-LDA will involve re-establishing the detection range and sensitivity of this method using superparamagnetic particles and more sensitive detection of amplicons with the MAR™ instrument. A method for isolation of viral RNA, which will be suitable for the proposed POC HIV-1 RNA assay, has to be investigated.

Future potential of converting this POC HIV RNA assay from a diagnostic into a monitoring assay is currently being considered.

Overall, this project addresses the challenges faced by South Africa in providing government laboratories with an appropriate technology for more affordable diagnostics and monitoring of HIV-1 in our population. An advanced knowledge in the field of molecular diagnostics has been acquired through this study. In addition, a partnership between academia and an international commercial company has been established. The in depth evaluation of different technologies described in this thesis show that the development of more affordable and appropriate technologies for diagnostic and monitoring of HIV-1 in a resource poor setting is an achievable goal.

Appendix A: Comparison of NAT based HIV-1 viral load assays

Table A1. Summary of the main characteristics of NAT based HIV-1 viral load assays.

Company	bioMerieux	bioMerieux	Bayer	Roche	Roche	Abbott	Abbott
Assay Name	**NucliSens HIV-1QT**	**NucliSens EasyQ**	**Bayer Versant HIV-1 Quantiplex v 3.0**	**COBAS Amplicor HIV-1 Monitor v1.5**	**COBAS Taqman**	**LcX HIV-1**	**RealTime HIV-1**
Assay Type	NASBA	NASBA	bDNA	RT-PCR	RT-PCR	RT-PCR	RT-PCR
Dynamic Range (copies/ml)	50-8 000 000	50-3 000 000	75-500 000	50-750 000	40-10 000 000	50-1 000 000	40-10 000 000
Specimen Type	Plasma, serum, DBS	Plasma, serum, DBS	Plasma	Plasma, DBS	Plasma	Plasma	Plasma
Specimen Volume (µl)	10-2000	10-2000	1000-2000	200-500	500	200-1000	1000
Area of Genome Targeted	*gag*	*gag*	*pol*	*gag*	*gag*	*pol*	*pol*
Subtype Reactivity	All	All	Group M (A-G)	All	All	Group M and O	Group M (B; C; F; B/F)
Time to result (hrs)	3.5	3.5	22-24	6	2	5	2-4
Tech Skill	High	High- Med, if automated	High-Med, if automated	High-Med, if automated	High-Med, if automated	High	High-Med
Lab Set up (PCR specifications required for all). Major Equipment	Extractor Biohazard hoods Easy Q Reader	Extractor Analyser Biohazard hoods	Bayer system 340, Centrifuge Heating Block Waterbath Biohazard hoods	COBAS Ampliprep Biohazard hoods Centrifuges Heating blocks Computer and printer Dead Air box	COBAS Taqman system Waterbath Heating block Biohazard hoods Centrifuges	Biohazard hoods Centrifuges Abbot LcX analyzer Thermalcycler Vacuum pump Heating block	Biohazard hoods Centrifuges Abbot *m2000sp* and *m2000rt* Computer and printer
Throughput (samples per run)	50	40	12-168	9-48	9-48	21	48-96
Cost/test, kit only (USD)	40-100	40-60	125	30-100	30-100	20-70	24-40

Appendix B: Polyacrylamide gel and buffers used for gel electrophoresis and DNA amplification

15% Acrylamide/8 M Urea Gel:

(For 15 ml, enough for a 13 cm x 15 cm x 0.75 mm thick gel):

- 7.2 g urea, high quality.
- 1.5 ml 10xTBE.
- 5.7 ml 40% acrylamide (acrylamide: bis acrylamide = 19:1).
- Adjust volume with dH_2O up to 15 ml.
- Stir at room temperature until urea has dissolved.

Add:

- 120 µl 10% ammonium persulfate in dH_2O.
- 16 µl TEMED
- Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour gel immediately.

50xTAE (Tris – Acetate EDTA, DNA electrophoresis buffer):

- Dissolve 242 g Tris in 500 ml dH_2O.
- Add 100 ml 0.5 M Na_2EDTA (pH 8.0) and 57.1 ml glacial acetic acid.
- Adjust volume to 1 liter with H_2O.
- Store at room temperature.

10xTBE Gel running buffer:

- 0.89 M Tris (109 g Tris base)
- 0.89 M Boric acid (55 g boric acid)

- 20 mM EDTA (40 ml 0.5 M EDTA, pH 8.0)
- Mix and store at 4ºC until use.

Potassium Phosphate buffer (pH 7.6):

- 1M Mono-salt (KH_2PO_4): 13.6 g $KH2PO_4$ dissolve in 50 ml H_2O; adjust volume to 100 ml with H_2O.
- 1 M Di-salt (K_2HPO_4): 17.4 g K_2HPO_4 dissolve in 50 ml H_2O and then adjust to 100m.l.
- Mix 6.5 ml of 1 M mono- and 43.5 ml of 1 M di-salt solutions with 100 ml H_2O. Check and adjust if necessary to pH 7.6. Make up final volume of the buffer to 200 ml with H_2O.

Appendix C: Composition of buffers and solutions used for cloning of DNA fragments

IPTG stock solution (0.1 M):

- 1.2 g IPTG (Promega cat # V3951)
- Add H_2O to 50 ml final volume. Filter-sterilize and store at 4ºC.

X-Gal (2 ml):

- 100 mg 5- bromo-4 chloro-3-indolyl-ß-D-galactoside (Promega Cat# V3941).
- Dissolve in 2 ml of N, N' – dimethyl- formamide. Cover with aluminum foil and store at - 20ºC.

LB (Luria - Bertani) Medium (per 1 liter):

- 10 g Bacto-tryptone
- 5g Bacto - yeast
- 5 g NaCl
- Adjust pH to 7.0 with NaOH.
- Autoclave.

LB plates with ampicillin:

- Add 15 g agar to 1 liter pf LB medium. Autoclave. Allow the medium to cool to 50ºC before adding ampicillin to a final concentration of 100µg/ml.
- Supplement with 0.5 mM IPTG and 80 µg/ml X-Gal.
- Pour 30-35 ml of medium into 85 mm Petri dishes. Let the agar harden and store at 4ºC for up to 1 month or at room temperature for up to 1 week.

SOC Medium (100 ml):

- 2.0 g Bacto – tryptone
- 0.5 g Bacto-yeast extract
- 1 ml 1 M NaCl
- 0.25 ml 1 M KCl
- 1 ml 2 M Mg^{2+} stock, filter-sterilized (as prepared below)
- 1 ml 2 M glucose, filter-sterilized.
- Add Bacto – tryptone, Bacto-yeast extract, NaCl and KCl to 97 ml of distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2 M Mg^{2+} stock and 2 M glucose, each to a final concentration of20mM. Bring to 100 ml with sterile, distilled water. Filter the complete medium through a 0.2μm filter unit. The final pH should be 7.0.

2 M Mg^{2+} stock:

- 20.33 g $MgCl \cdot 6\ H_2O$
- 24.65 g $Mg\ SO_4\ 7\ H_2O$
- Add distilled water to 100 ml. Filter sterilize.

Appendix D: Ethics Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Scott & Student/s

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M00-01-07

PROJECT	Research & Development Programme, Department of Molecular Medicine And Haematology
	Class approval valid until 2008
INVESTIGATORS	L Scott & Student/s
DEPARTMENT	Molecular Medicine & Haem., NHLS
DATE CONSIDERED	00/28/01

DECISION OF THE COMMITTEE *

Within this class approval falls N Rekhviashvili
Drs G Stevens & W Steven ' Cost Effective Diagnosis
& Monitoring of HIV-1 in a Resource Poor Setting

DATE 03-04-27 CHAIRMAN.....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr L Scott
Dept of Molecular Medicine & Haem.., NHLS

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 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.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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