Evaluation and Design

of

Affordable and Novel

HIV-1 Drug Resistance Assays:

with reference to mutations arising from 2 nucleoside reverse transcriptase inhibitors, Stavudine & Didanosine, & one non-nucleoside reverse transcriptase inhibitor, Efavirenz.

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

Master of Science in Medicine

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DECLARATION

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

Carole Wallis

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……9……..day of…May……..2005

DEDICATION

This dissertation is dedicated to my parents, Norman and Shirley Wallis, whom I have learnt and, been inspired to do, many things. From them I have learnt that a journey begins not with the first step but with the desire to go where you have never gone before, and that real education begins with the willingness to learn from mistakes. These two lessons have stood me in good stead through this dissertation.

PUBLICATIONS & PRESENTATIONS ARISING FROM THIS STUDY

Publications:

Papers:

1. **Carole Lorraine Wallis**, Imraan Mahomed, Lynn Morris, Thato Chidarikire, Gwynn Stevens, Natela Rekhviashvili and Wendy Stevens (2005). Evaluation of an Oligonucleotide Ligation Assay (OLA) for detection of mutations in HIV-1 subtype C individuals who have high level resistance to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. Journal of Virological Methods, May; 125(2):99-109. (Appendix F)

Conference presentations:

1. **C Wallis**, T Chidarikire, G Stevens, N Rekhviashvili, and W Stevens. Evaluation of an Oligonucleotide Ligation Assay (OLA) for detection of NNRTI mutations in HIV-1 subtype C. 2nd European HIV drug resistance workshop, 2004, Rome.
2. I. Mahomed, **C Wallis**, N Rekhviashvili, G Stevens and W Stevens. Evaluation of the Oligonucleotide Ligation Assay (OLA) in HIV-1 subtype C. WITS Faculty of Health Science Research Day, 2004, Johannesburg
3. **C Wallis**, N Rekhviashvili, G Stevens, W Stevens. A rapid and affordable assay for the detection of the V106M mutation associated with HIV-1 drug resistance to all known non-nucleoside reverse transcriptase inhibitors. 2nd South African AIDS Conference, 2005, Durban.

ABSTRACT

Approximately 5 million individuals are infected with HIV/AIDS in South Africa. The South African government has initiated a National Anti-retroviral therapy (ARV) Program to manage this disease. The emergence of drug resistance to ARV therapy is of great concern. Commercial gold standard sequence-based genotyping assays for monitoring resistance are unaffordable.

This project aimed at developing affordable methods to detect specific point mutations relevant to HIV-1 subtype C. The Oligonucleotide Ligation assay (OLA), a real-time PCR assay and a Restriction Fragment Length Polymorphism (RFLP) assay were explored. Results were compared to the Viroseq genotyping assay. OLA performed poorly on HIV-1 subtype C samples and needs modification. The real-time PCR assay using short Minor Groove Binding probes, accurately detected the K65R mutation. The *Mae III* RFLP assay detected all V106M mutations accurately. Longitudinal cohort studies are required to confirm relevant mutations, appropriate assays and algorithms for resistance monitoring in HIV-1 subtype C.

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- Lastly I would like to thank the staff of the department of Molecular Medicine and Haematology for their encouragement and interest in my work, particularly Dr Maria Papathanasopoulos and Dr Lesley Scott.

TABLE OF CONTENTS **PAGE**

LIST OF FIGURES **PAGE**

LIST OF TABLES **PAGE**

NOMENCLATURE

3TC=Lamivudine

ABC= Abacavir

AIDS = Acquired Immunodeficiency syndrome

APV=Amprenavir

ART=antiretroviral therapy

ARV=antiretroviral

ATP=Adenosine triphosphate

AZT=Zidovudine

bp=base pairs

BSA=Bovine Serum Albumin

CA=capsid

CD = clusters of differentiation

cDNA=complementary deoxyribonucleic acid

CIPRA=Comprehensive International Program of Research on AIDS-South Africa

CLS=Contract Laboratory Services

d4T=Stavudine

ddc=Zalcitabine

ddI=Didanosine

DLV=Delavirdine

DNA=deoxyribonucleic acid

dNTP= deoxynucleoside triphosphate

EDTA=Ethylenediaminetetra-acetic acid

EFV=Efavirenz

EGG=Euro-Guidelines Group

ELISA=Enzyme Linked Immunosorbent Assay

ENF=Enfuvirtide (T-20)

FDA=Food and Drug Administration

FPV=Fosamprenavir

FTC= Amtricitabine

Gp=glycoprotein

HAART=highly active ART

HIV=human immunodeficiency virus

IDV=Indinavir

IN=integrase

IAS-USA=International AIDS society of the United States of America

kb=kilobases

KCl=Potassium Chloride

LiPa=Line Probe Assay

LPV=Lopinavir/Ritonavir

MA=matrix

MDR=multi-drug resistance

MGB=Minor Groove Binding

$MgCl_2$=Magnesium chloride

µl=microlitre

ml=milliliter

MTCT=mother to child transmission

MuLV=Murine Moloney Virus

MW= molecular weight

NaCl=Sodium Chloride

NaOH=Sodium Hydroxide

NC=nucleocapsid

NFV=Nelfinavir mesylate

ng=nanogram

NHLS=National Health Laboratory Service

NICD=National Institute for Communicable Diseases

NIH=National Institute of Health, USA.

Nm=nanomole

NNRTI=Non-nucleoside

NRTI=Nucleoside Reverse Transcriptase Inhibitors

NtRTI=Nucleotide Reverse Transcriptase Inhibitors

NVP=nevirapine

OD=Optical densities

OLA=Oligonucleotide Ligation Assay

p17=protein 17

PBMC=Peripheral Blood Mononuclear Cells

PCR= polymerase chain reaction

PI=Protease Inhibitors

PR=protease

PSA=Phosphate Buffer Saline

RFLP=Restriction Fragment Length Polymorphism

RNA=ribonucleic acid

RT=reverse transcriptase enzyme

RTI=Reverse Transcriptase Inhibitors

RTV=Ritonavir

SA=South Africa

SGS=single-genome sequencing

SQV=Saquinavir meslate

SD=standard deviation

SU=Surface

T-20=Enfuvirtide

TEA=Tris Ethylenediaminetetra

TAMs=Thymidine Analogue Mutations

TDF=Tenofovir

TM=transmembrane

TMB=3,3',5,5'-Tetra-methyl-benzidine

UK=United Kingdom

US=United States

USA=United States of America

UV=ultraviolet

WITS=University of the Witwatersrand

WHO=World Health Organisation

VQA=Virology Quality Assurance

PREFACE

Several events initiated and steered the direction of this study. The South Africa government has now accepted the impact of the HIV/AIDS catastrophe and to manage this disease, Antiretrovial (ARV) therapy is now starting to be prescribed in the public sector. To support these treatment programs, affordable and appropriate laboratory monitoring is essential.

1. CHAPTER ONE: INTRODUCTION

1.1. Introduction to HIV-1 resistance testing in developing countries.

The Human Immunodeficiency Virus (HIV) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) in humans, which is characterized by the depletion of CD4 positive lymphocytes (Abacioglu, et al., 1994). HIV-1 has reached pandemic proportions with more than 39.4 million people estimated to be infected with the virus by December 2004 with 4.9 million people newly infected in 2004 (UNAIDS, 2004). Approximately 3.1 million people are thought to have died from AIDS in 2004. The disease continues to affect humans living in all geographic areas, but the most affected area is Sub-Saharan Africa, which has approximately 25.4 million people infected (UNAIDS, 2004). Currently there is no preventative vaccine or cure for HIV/AIDS, however, there are antiretroviral (ARV) drugs which slow down the rate of disease progression.

Over 10% of the world's population lives in resource poor sub-Saharan Africa and at least 60% of HIV-1 infected individuals live in this region (UNAIDS, 2004). South Africa has the highest number of people living with HIV-1 in the world, with an estimated 5.3 million infected (Dorrington, 2002). Meehan et al., (2004) performed surveillance studies in South Africa which indicated that approximately 40% of young women are HIV-1 positive. Moreover, it is estimated that 600 people in South Africa die from an HIV related illness each day (African Action, 2003).

In an attempt to manage this pandemic, there has been increased global support to fund preventative and treatment programs, and reduce drug costs (Brown, 2000; Gottlieb, 2001; Sidley, 1998; Sidley, 2000; Sidley, 2001; Yamey, 2000). Furthermore the World Health

Organisation (WHO) and UNAIDS have activated the 3 by 5 initiative, a global initiative aimed at treating 3 million HIV-1 infected people in resource poor sub-Saharan Africa, with antiretroviral therapy (ART) by 2005. To parallel these clinical initiatives laboratory monitoring is critical and the cost thereof remains high (Tucker and Yeats, 2001). This study deals with the need for affordable resistance testing, a critical component of monitoring of ARV therapy.

1.2. Introduction to HIV

HIV is a retrovirus and contains its genetic information in the form of single stranded RNA. These viruses are able to reverse transcribe their RNA into cDNA using their reverse transcriptase enzyme (RT), following which cDNA integrates into the host's DNA upon cell infection.

1.2.1. HIV Structure:

HIV is spherical in shape and approximately 80-130nm in diameter. The virus is made up of two major portions, the envelope and the core. The envelope consists of a lipid bilayer derived from the human cell membranes during viral budding (Collier, et al., 1999). Embedded in this bilayer are viral derived proteins: the transmembrane envelope glycoprotein 41 (gp41; trimeric configuration), which connect the gp120 trimeric surface knobs to the membrane. Underlying the envelope is the matrix protein layer formed by the protein 17 (p17). The cone shaped viral core is composed of the capsid (p24) (Gelderblom, et al., 1989), which surrounds two single stranded RNA molecules, tightly covered by the nucleocapsid protein p7. The enzymes protease (PR), RT and integrase (IN) are also located within the capsid (Figure 1.1).

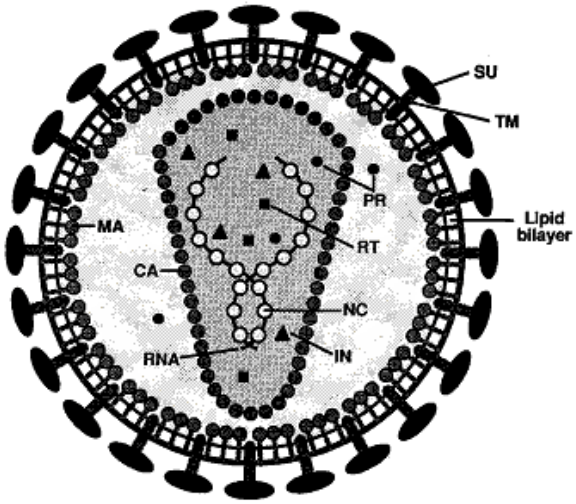


Figure 1.1: Cross sectional schematic representation of the structure of HIV. SU, Surface gp120; TM, transmembrane gp41; MA, matrix p17; CA, capsid p24; NC, nucleocapsid p7; PR, protease; RT, reverse transcriptase and IN, integrase (Volker, 1993).

1.2.2. HIV Life Cycle:

HIV infects T-lymphocytes and/or macrophages that express the receptor CD4 on their surface (Dalgleish, et al., 1984; Klatzmann, et al., 1984). Viral infection is initiated when the gp120 recognizes specific binding sites on the CD4 receptor (King, 1994). The gp120-CD4 interaction results in a conformational change in gp120 which exposes the co-receptor binding sites, either CXCR4 or CCR5 (Alkhatib, et al., 1996; Dragic, et al., 1996). Co-receptor binding induces further conformational changes in gp120 allowing the 6 helix bundle to form in gp41. This leads to fusion between the viral and cellular membranes, allowing the HIV core to enter the host's cytoplasm (Turner & Summers, 1999; Figure 1.2). An uncoating step leads to release of the viral RNA and essential enzymes from the capsid core. The protease enzyme cleaves the p7 protein from the RNA allowing the RT to reverse transcribe the RNA into cDNA and into double stranded DNA. This is transported to the nucleus, where the IN facilitates the integration of viral DNA into genomic DNA. The proviral DNA is used to synthesize viral proteins using the host's replication

machinery and assembled into immature virions, which are released by budding through the infected cell membrane (Figure 1.2). Released virions are matured by the protease enzyme (Volker, 1993; Turner and Summers, 1999). Each step of the viral life cycle is a potential drug target. Drugs on the market currently target the RT, PR enzyme and viral entry.

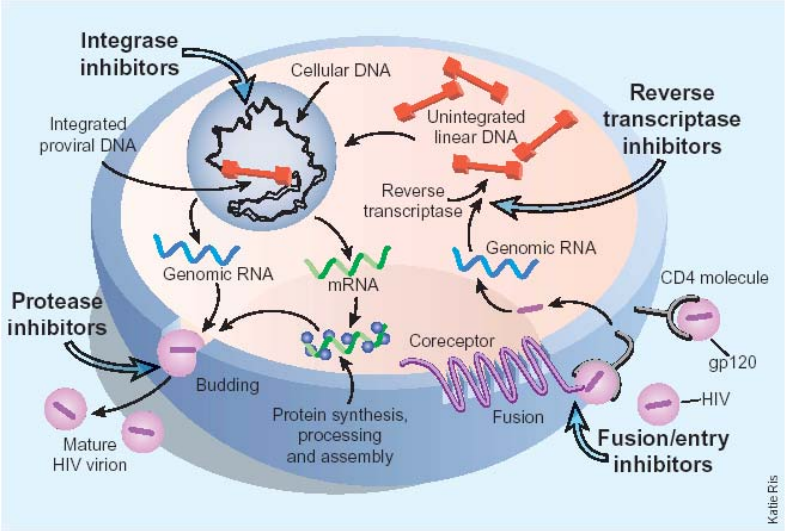


Figure 1.2: The life cycle of the HIV-1 virus and the targets for drug therapy. The HIV lifecycle is shown here from cell fusion, to integration into genomic DNA and virion assembly and release. The 4 drug target areas are shown here: Reverse Transcriptase Inhibitors; Protease Inhibitors; Integrase Inhibitors and Entry Inhibitors (Fauci, 2003).

The HIV genome encodes 3 structural genes (*gag, pol* and *env*), 2 regulatory genes (*tat* and *rev*), and 4 accessory genes (*vif, vpr, vpu* and *nef*) (Figure 1.3). The viral genome is approximately 10 kilobases (kb) in size and contains open reading frames for at least 18 proteins. The $pr160^{gag\text{-}pol}$ polyprotein is cleaved by the viral PR into 9 subunits, PR; RT which contains RNAse H; integrase (IN); matrix p17 (MA); capsid p24 (CA); p2; nucleocapsid p7 (NC); p1 and p6 (Figure 1.3). The RT heterodimer comprises the p51 subunit (RNAse H; represents the first 440 amino acids of the *pol* gene), and the p66 subunit (RT; represents all 560 amino acids of the *pol* gene), resulting in 440 amino acids

common to both subunits. The p66 subunit makes up the DNA binding groove and the active site, and resembles an open hand, whereas p51 has shown to have no known enzymatic activity (Larder and Stammers, 1999). The envelope glycoprotein gp160 precursor is cleaved by the host's cellular proteases into the gp120 and gp41 subunits.

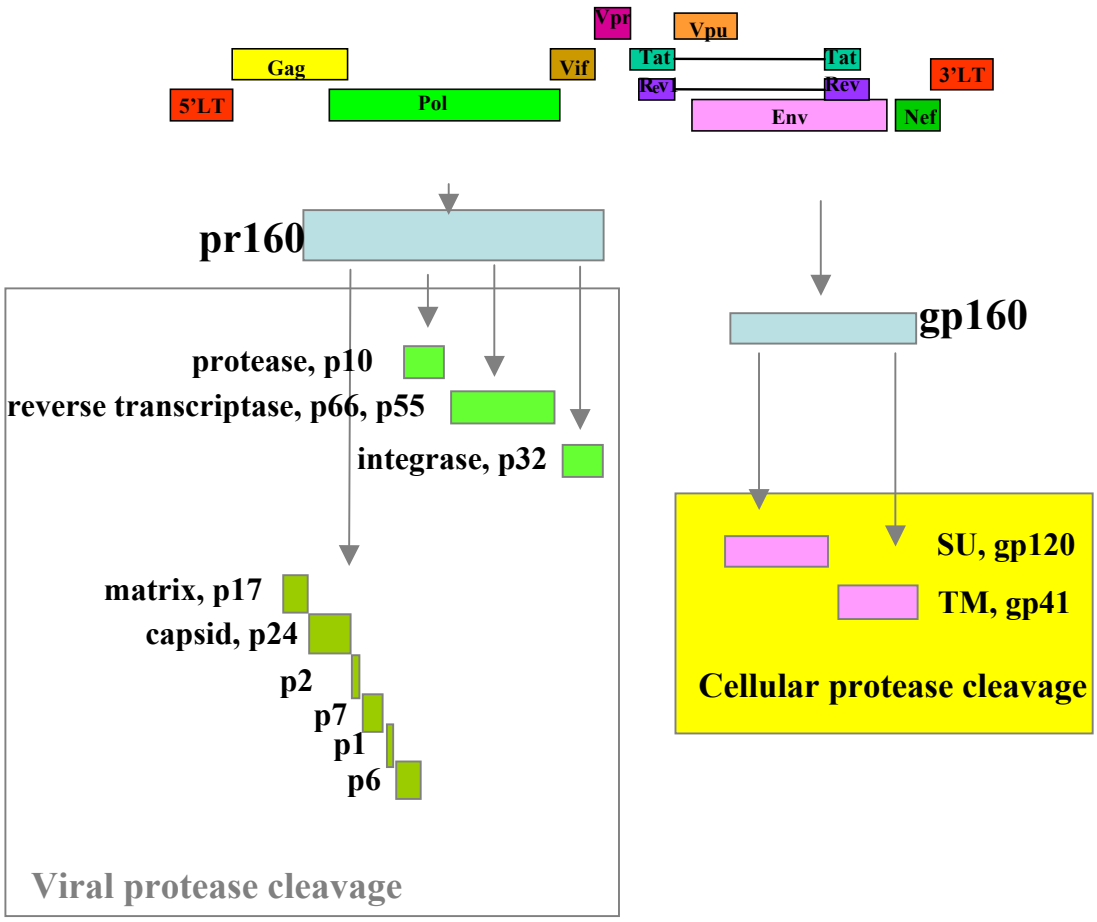


Figure 1.3: The HIV genome and proteins (Adapted from Peterlin and Trono, 2003, Nature Reviews).

1.3. Implications of the HIV Replication Strategy:

During each replication cycle HIV generates several variants and quasi-species as a direct result of poor proof-reading by RT (DeGruttola, et al., 2000). This poor proofreading results in point mutations, insertions and deletions, which occur up to 10^4-10^5 times per

day in an infected host (Boyer, et al., 1992; Coffin, 1995). This process is further amplified by the high level of virus production, 10^{10} virions per day (Coffin, 1995). This replication strategy has resulted in a highly variable and constantly evolving virus, which impacts on factors such as viral diversity, immune escape and ARV drug resistance (Mansky, 1998).

1.3.1. HIV Diversity:

There are two distinct types of HIV, HIV-1 and 2. These two viruses are distinguished by their genome organization and phylogenetic differences (Los Alamos HIV Database, 2001). The ongoing evolution of HIV-1 and 2 has resulted in substantial genetic diversity amongst different isolates. HIV-2 is classified into 5 groups, A, B, C, F and G. HIV-1 is classified into 3 distinct groups by looking at phylogenetic analysis. (Los Alamos HIV Database, 2001). These 3 distinct groups are referred to as: M for Major, O for Outlier and N for non-M or non-O. Group O appears in west and central Africa, and N was only discovered in 1998 and appears limited to Cameroon. More than 90% of HIV infections worldwide belong to HIV-1 group M. Group M is the major group and has further been divided into 9 subtypes A, B, C, D, F, G, H, J and K, 2 sub-subtypes A1, A2, A3 and F1, F2 and 16 circulating recombinant forms (Los Alamos HIV Database, 2001). Subtypes appear to have specific geographic distributions. The predominant subtype in the United States and Europe is subtype B, whereas in Africa, subtype A and G occur mainly in Central and West Africa and subtype C is the pre-dominant subtype in Southern Africa (Papathanasopoulos, et al., 2003). Subtype C is the most rapidly expanding subtype and accounts for over 50% of new infections worldwide. The genetic diversity of HIV-1/2 represents a significant challenge to disease management.

1.3.2. HIV Drug Resistance:

The error prone replication rate of HIV-1 allows the virus to escape the host's immune system, thereby making the host incapable of controlling the virus. In the absence of a vaccine, the only way to manage this virus is with ARV drugs. However, HIV's genetic variation results in incomplete suppression of viral replication, allowing the emergence of HIV-1 drug resistant strains (Leitner, et al., 2003).

1.4. Disease progression and management of HIV-1 infection:

The natural history of HIV-1 infection and disease progression to AIDS is shown in figure 1.4a. Briefly, after primary HIV-1 infection, the host manages to control viral replication (viral load) at a set point and maintains $CD4^+$ cell counts for a number of years (asymptomatic phase). At some critical point the $CD4^+$ cells are severely depleted and viral load increases dramatically. The infected individual becomes susceptible to opportunistic diseases and is diagnosed with AIDS. In the attempt to prevent the progression to AIDS, highly active antiretroviral therapy (HAART) has been introduced. Successful HAART can effectively suppress viral replication and reduce the viral load to <50copies/ml (Figure 1.4b) within an average of 12 weeks (Fauci et al., 1996). However, treatment failure is common and thought to occur in 10% of patients within the first year of treatment (per.comm. Dr F Venter). Emergence of drug resistant HIV is thus an inevitable consequence of the failure to fully suppress viral replication in these individuals.

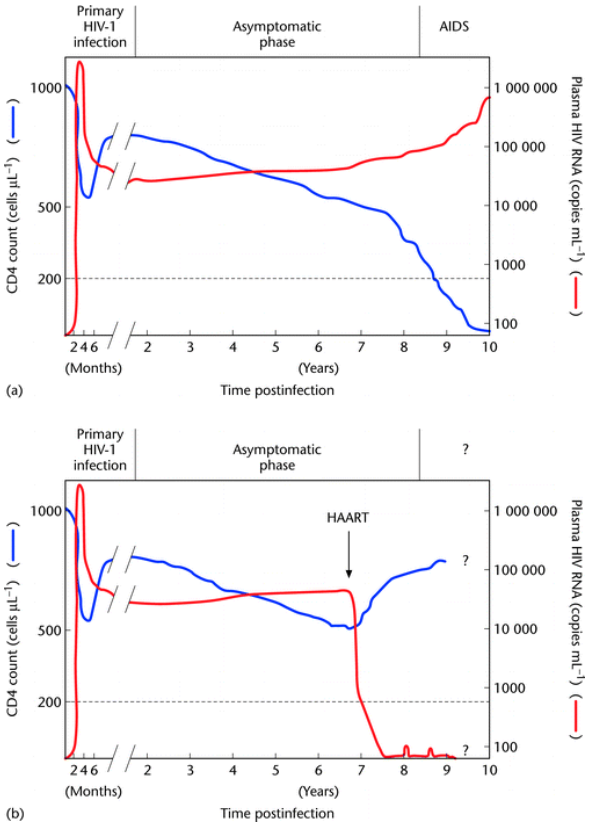


Figure 1.4: The natural history of HIV infection and AIDS in untreated (a) and treated (b) individuals. (Adapted from Fauci et al., 1996)

In developed countries the criteria to initiate HAART are a CD4 count <350cells/ml or a viral load >55000 copies/ml (Dybul, et al., 2002). However, in resource poor settings like South Africa the eligibility for enrolment include a CD4 count <200 and/or stage 4 clinical diseases as described in the WHO clinical grading scheme (The WHO International collaborating Group for the study of the WHO staging system, 1993).

1.5. Treatment Failure:

Treatment failure on HAART can result from a combination of factors. Firstly, drug resistance mutations may pre-exist and are selected for under drug pressure (Lazzari, et al., 2004). Secondly, there may be sub-inhibitory drug levels which result in inhibition of drug

susceptible virus, but allow for the outgrowth of resistant strains. Sub-inhibitory drug levels may arise as a result of several different events including poor patient adherence, inadequate drug absorption, insufficient drug dosage and interactions between different drugs. Pharmacokinetic variability may also result in the development of drug resistance, since circulating drug levels can influence suppression of viral replication. There are four main cellular mechanisms which can affect pharmacokinetic variability; a) cytochromes- a family of enzymes abundant in intestinal epithelial cells and the liver, these enzymes metabolize certain drugs like Reverse Transcriptase Inhibitors (RTI) and Protease Inhibitors (PI); b) P-glycoprotein which is a cell membrane protein that moves drugs from the epithelium wall back into the lumen of the intestine thereby regulating drug concentration in the plasma; c) certain individuals have a multi-drug resistance (MDR) protein which confers resistance to certain drugs and this can result in sub-optimal drug levels in the plasma; d) cellular kinases which are required in drug phosphorylation, and have a direct influence on plasma drug concentration (Miller, 2001). The selective pressure exerted by the drugs may also favour the evolution of drug resistance mutations; and drug induced toxicities result in poor adherence.

1.6. Food and Drug Administration (FDA) approved antiretroviral drugs:

Currently there are more than 20 FDA approved antiretroviral drugs (ARV) for use in HIV infection; which fall into 3 main classes: Reverse Transcriptase Inhibitors (RTI), Protease Inhibitors (PI) and Entry Inhibitors (Fauci, 2003). Genetic counterparts are also available, but have not been fully evaluated.

Table 1.1: Currently available FDA approved ARV drugs. Highlighted drugs indicate the regimens planned for the South African (SA) national roll-out program.

Class	Brand Name	Generic Name	Abbreviation	Pharmaceutical Company
NRTIs	Combivir	Zidovudine, Lamivudine	AZT + 3TC	GlaxoSmithKline
	Emtriva	Amtricitabine	FTC	Gilead Sciences
	Epivir	Lamivudine	3TC	GlaxoSmithKline
	Epzicom	Abacavir, Lamivudine	ABC + 3TC	GlaxoSmithKline
	Hivid	Zalcitabine	ddC	Roche
	Retrovir	Zidovudine	AZT/ ZDV	GlaxoSmithKline
	Trizivir	Abacavir, Zidovudine & Lamivudine	ABC + AZT +3TC	
	Truvada	Tenofovir and Emtricitabine	TDF + FTC	Gilead Sciences
	Videx	Didanosine	ddI	Bristol-Myers Squibb
	Videx EC	Didanosine: delayed-release capsules	ddI	Bristol-Myers Squibb
	Zerit	Stavudine	d4T	Bristol-Myers Squibb
	Zerit XR	Stavudine: delayed-released	d4T	Bristol-Myers Squibb
	Ziagen	Abacavir	ABC	GlaxoSmithKline
NtRTI	Viread	Tenofovir disoproxil fumarate (DF)	TDF	Gilead Sciences
NNRTI	Rescriptor	Delavirdine	DLV	Pfizer
	Sustiva	Efavirenz	EFV	Bristol-Myers Squibb
	Viramune	Nevirapine	NVP	Boehringer Ingelheim
PI	Agenerase	Amprenavir	APV	GlaxoSmithKline
	Crixivan sulfate	Indinavir	IDV	MERCK
	Fortovase	Saquinavir meslate (soft gel cap)	SQV(SGC)	Roche
	Invirase	Saquinavir meslate (hard gel cap)	SQV(HGC)	Roche
	Kaletra	Lopinavir/Ritonavir	LPV	Abbott Laboratories
	Lexiva	Fosamprenavir calcium	FPV	GlaxoSmithKline
	Norvir	Ritonavir	RTV	Abbott Laboratories
	Reyataz	Atazanavir sulfate	ATZ	Bristol-Myers Squibb
	Viracept	Nelfinavir mesylate	NFV	Agouron Pharmaceuticals
Entry Inhibitor	Fuzeon (T-20)	Enfuvirtide	ENF	Roche

1.6.1. Reverse Transcriptase Inhibitors (RTI):

This group of inhibitors is important as they are affordable and are the backbone of most ARV therapeutic regimens. They are comprised of Nucleoside RT Inhibitors (NRTIs), Nucleotide RT Inhibitors (NtRTIs) and Non-NRTI (NNRTIs).

1.6.1.1. NRTIs and NtRTIs

These drugs act as nucleoside/tide bases, but are chemically modified to cause chain termination. Chain termination occurs because these modified bases lack a hydroxyl group at position 3' of their sugar moiety (Clavel, et al., 2004). Prior to recognition by the RT enzyme, these bases need to be transformed into triphosphate derivatives, and are incorporated into the growing DNA strand resulting in chain termination (Figure 5a). NRTIs are specific to the HIV RT enzyme and poorly recognized by other polymerases in the cell. The drugs classified in this group differ in chemical structure and mimic different nucleosides/tides. For example, Zidovudine (AZT) and Stavudine (d4T) are both thymine analogues; Didanosine (ddI) is an adenine analogue; Zalcitabine (ddC) and Lamivudine (3TC) are cytosine analogues and Abacavir (ABC) is a guanine analogue. Tenofivir and Adefovir are unique acyclic structures with a phosphate group already attached and are able to surpass the initial phosphorylation step (Miller, 2001 and Clavel, et al., 2004).

Resistance to NRTIs is caused by a single and/or combination of mutation(s), which can arise by two different mechanisms. Firstly, the mutations result in the RT enzyme 'choosing' the non-modified base rather than the modified one, thereby ensuring chain elongation (Huang, et al., 1998; Larder and Stammers 1999). Secondly, the mutated RT enzyme can remove the chain terminating dNTPs by ATP hydrolysis (Shafer, 2004).

1.6.1.2. NNRTIs

These drug molecules bind directly to the hydrophobic pocket in the RT enzyme located between the 2 Beta (β) sheets of the p66 subunit. Residues from the p51 subunit complete a small portion of this drug binding pocket. This pocket is located close to the active site

of the RT enzyme. NNRTIs inhibit replication by allosterically displacing the catalytic aspartate residues close to the polymerase-binding site (Kohlstaedt, et al., 1992), resulting in blocking of DNA polymerization (Figure 1.5b). This hydrophobic pocket has been found to be less conserved amongst different subtypes compared to the dNTP-binding site used by NRTIs, resulting in differing responses to the NNRTIs. For example, data has shown that HIV-1 group O isolates are naturally resistant to NNRTIs (Descamps, et al., 1997).

NNRTI resistance arises when a single mutation occurs in the poorly conserved hydrophobic pocket of the RT enzyme, changing its physical properties. These changes result in poor drug binding. Sequence variability results in poor viral suppression causing rapid resistance to NNRTIs (Jackson, et al., 2000). The 3 known NNRTIs target the same region of this pocket, so one mutation can cause cross-resistance to all these drugs.

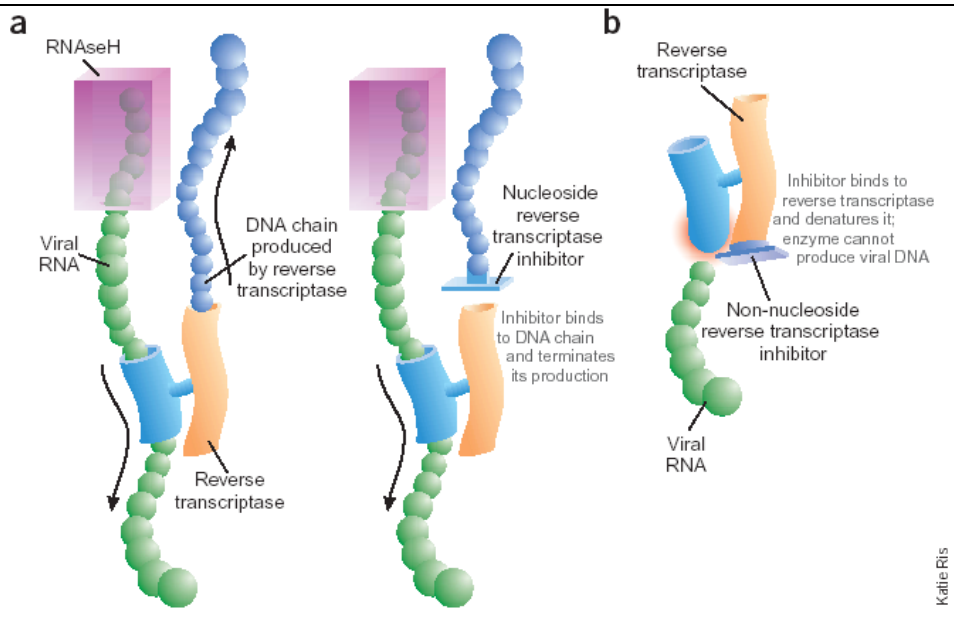


Figure 1.5: (**a**) NRTIs prevent replication by inhibiting the RT enzyme. This results in chain termination and, (**b**) NNRTIs bind to a site distant from the active site of the RT enzyme. This causes a conformational change of the RT enzyme and inhibits replication (Pomerantz and Horn, 2003).

1.6.2. *Protease Inhibitors(PI)*

Protease inhibitors irreversibly inhibit the protease enzyme by binding to its active site. This enzyme plays a vital role in the viral life cycle and is responsible for assisting in maturation of the viral particle. In the absence of the protease enzyme, long polypeptides are not cut into smaller pieces to create functional and mature proteins (Miller, 2001) (Figure 1.6). These drugs specifically target the HIV-1 protease enzyme and do not affect other proteases in the human cell. Novel PIs being evaluated target multi-drug resistant strains and focus on a conserved area of the protease enzymes active site (Gulnik, et al., 2004). Interestingly, certain HIV-1 subtype G strains are less susceptible to PIs (Papathanasopoulos, et al., 2003).

Resistance to PIs can arise from mutations at three different sites. Firstly, mutations arising at the active site of the protease enzyme can change its physical properties. This results in poor protease inhibitor binding and no viral suppression. Secondly, mutations can arise a distance from the active site. These mutations cause resistance by changing the kinetics of the enzyme or by increasing the protease's enzymatic properties thereby enhancing viral replication. Lastly, mutations can arise in *gag* and the *gag-pol* cleavage sites, which decrease the processing of the polypeptide.

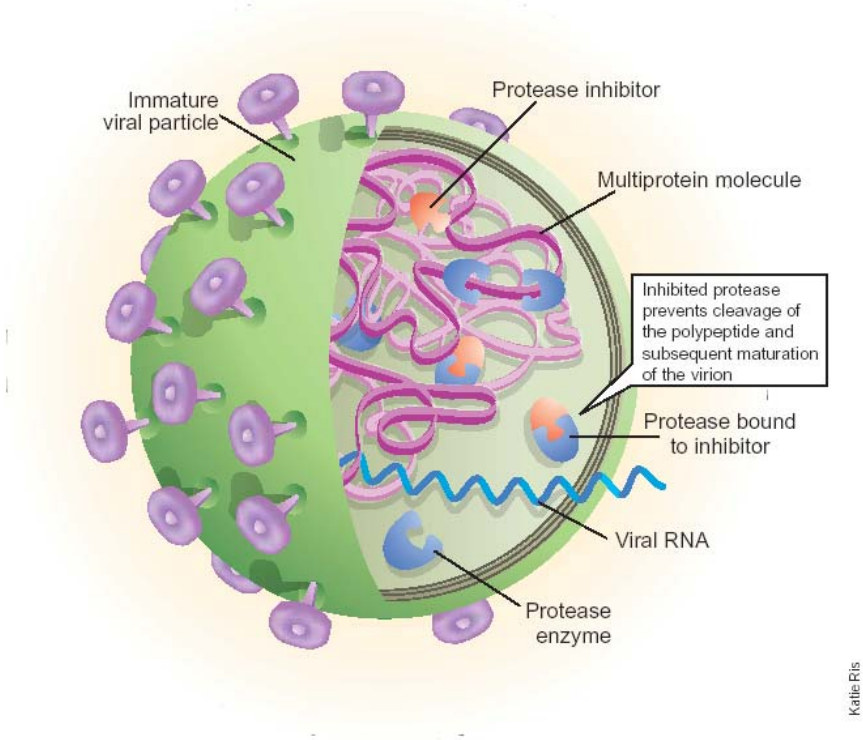


Figure 1.6: Protease Inhibitors bind to the active site of the protease enzyme, rendering it inactive. This results in the protease enzyme being unable to cleave the *gag-pol* polypeptide chain into smaller pieces to create functional and mature proteins (Pomerantz and Horn, 2003).

1.6.3. Entry Inhibitors:

Entry inhibitors work by preventing viral entry into the cell. There is currently one entry inhibitor that is FDA approved: Enfuvirtide (T-20). T-20 is a 36 amino acid peptide, which was derived from the HR2 sequence of HIV-1_{LAI} subtype B gp41 envelope glycoprotein (Wild, et al., 1994). T-20 prevents the virus from entering the cell by binding to the HR2 region of gp41 and interferes with the formation of the six-helical bundle (Wild, et al., 1994; Kilby, et al., 1998). Novel entry inhibitors under evaluation target entry of the virus by interfering with the binding of the virus to CD4 (attachment inhibitors), CCR5 and/or CXCR4 (co-receptor inhibitors) and membrane fusion (Clavel, et al., 2004).

Several studies have investigated the development of resistance to T-20. Results from the TORO trial concluded that 90% of resistance to T-20 is a result of mutations occurring in the 36 to 45 amino acid region of gp41 (Rimsky, et al., 1998; Sista, et al., 2002; Wei, et al., 2002). These mutations result in T-20 being unable to bind to HR2 and interferes with the formation of the six-helical bundle.

1.7. Global ARV resistance patterns:

Several studies have shown that the pressure of certain ARV drugs results in the emergence of specific ARV resistance patterns. This allows for certain mutations to be predicted for by clinicians treating patients with HAART. An extensive list of all known mutations can be found at the Los Alamos HIV database (2001). As of May 2004 there were 791 HIV-1 mutations listed for various drug combinations, of which 28 occurred in Gag, 253 in Protease, 3 in Integrase, 320 in RT, and 187 in the Envelope regions. These mutations have predominantly been described in HIV-1 subtype B. For the purposes of this study, the classic mutations associated with the following RT drugs: Stavudine, Didanosine and Nevirapine or Efavirenz, are discussed in more detail (based on the drug regimen to be used in the ARV roll-out Program in South Africa).

Table 1.2: The mutations in the RT enzyme associated with HIV-1 resistance to d4T; ddI; NVP/EFV (D'Aquila, et al., 2002).

Drug	Abbreviation	Class	Mutations
Stavudine	d4T	NRTI	M41L, E44D, D67N, K70R, V118I, L210W, T215Y/F, K219Q/E
Didanosine	ddI	NRTI	K65R, L74V
Nevirapine/ Efavirenz	NVP/EFV	NNRTI	L100I, K103N, V106A (NVP), V108I, Y181C/I, Y181C/L, G190A

1.7.1. Stavudine (d4T):

Stavudine is a thymidine analogue and most mutations associated with this drug are those that result in direct discrimination of this base known as Thymidine Analogue Mutations (TAMS), namely M41L, D67N, K70R, L210W, T215Y/F, K219Q/E (Boucher, et al., 1992; Richman, et al., 2001). The E44D and V118I mutations are less common, and prevent chain termination in two different ways: E44D causes the modified nucleotide to be removed, whereas V118I helps in base discrimination (Perno, et al., 2001; Girouard, et al., 2003). However, all the above-mentioned mutations have been shown to occur mainly in regimens lacking NNRTIs (Miller, et al., 2002). A recent study, Gilead 903, demonstrated that when d4T was administered with an NNRTI (like EFV), no TAMS occurred. Interestingly, 2 subjects from this study developed the K65R mutation (a ddI associated mutation; Staszewski, et al., 2003). This is of particular interest since the regimen for the South African national roll-out program incorporates both d4T and EFV and ddI.

1.7.2. Didanosine (ddI):

Didanosine drug resistance is found to be associated with 2 mutations, namely, L74V and K65R. L74V is found to occur commonly during ddI monotherapy conferring a two to five-fold increase in resistance (Kozal, et al., 1994; Shafer, et al., 1994). However, it is rarely seen in combination with d4T (Pellegrin, et al., 1999; Coakley, et al., 2002). K65R's codon forms a loop between the β2 and β3 strands in the 'fingers' region of the RT enzyme, which are important during polymerization of the DNA strands. This codon directly interacts with the gamma phosphate of the bound dNTP. The mutation at this site causes resistance by allowing the discrimination between natural and modified dNTPs.

The incidence of the K65R mutation has recently been found to have increased in HAART patients from 1 to 4% (Miller, et al., 2003). Frankel, et al., (2004) showed that the presence of the K65R and L74V mutations decrease viral replication, RT processivity and results in less error prone replication.

1.7.3. Nevirapine (NVP) and Efavirenz (EFV)

NVP and/or EFV have been used in combination with NRTIs and studies have demonstrated that 50-70% of patients develop drug resistance because they harbour the K103N and/or Y181C mutations (Miller, et al., 2002; Squires, et al., 2002; Ferrer, et al., 2003). Longitudinal follow-up studies of women who received a single-dose of NVP in a perinatal HIV prevention program (HIVNET 012) have identified the emergence of NNRTI-resistant virus harbouring the K103N and/or Y181C mutations in at least 25% of participants (Eshleman, et al., 2004a). Codon 103 is located in the outer rim of the hydrophobic pocket of RT. K103N does not alter the RT; but when un-liganded allows the RT to stabilize the pocket by forming a network of hydrogen bonds, which inhibit NNRTI binding (Hsiou, et al., 2001). Moreover, K103N, causes a 20-50 fold increase in resistance to all NNRTIs (Young, et al., 1995; Huang, et al., 1999; Bacheler, et al., 2001). The presence of Y181C causes a 30 fold increase in resistance to NVP and DLV and a three fold increase in resistance to EFV (Shafer, 2004). Furthermore, in the presence of Y181C there can be reduced susceptibility to d4T (Parkin, et al., 2003, Baldanti, et al., 2003). The mutation V106A occurs in NVP regimens (Shafer, 2004). V106A results in resistance by ensuring the drug molecule cannot bind to the pocket, since codon 106 is situated inside the hydrophobic pocket (Shafer, 2004).

1.8. Current ARV resistance patterns in SA:

HIV/AIDS patients in the public and private sectors in South Africa have had limited access to ARVs, and a limited time to develop ARV drug resistance mutations (Morris, et al., 2000, Kantor, et al., 2002; Gordon, et al., 2003). Thus, limited ARV resistance data is available. Furthermore, the ARV mutations that occur in HIV-1 subtype C isolates have not been extensively described, and may be different from those in HIV-1 subtype B. ARV resistance studies performed so far reveal that HIV-1 subtype C individuals failing therapy in Zimbabwe develop similar mutation profiles to subtype B infected individuals failing therapy (Kantor, et al., 2002).

Unpublished data from South African mother-to-child transmission (MTCT) prevention programs have shown that NVP resistance profiles of HIV-1 subtype C viruses from mother and child pairs are different after single-dose NVP to mother and/or child (L. Morris, pers. comm.). The same group investigated the baseline mutations present in drug naïve patients, and compared the profiles to patients on mainly RT inhibitor regimens. Data showed that few mutations were present at baseline (at positions 98, 118 and 179 in RT), however ARV treated patients harboured a range of potential primary and secondary mutations associated with development of drug resistance (Figure 1.7). A comparison of drug resistance profiles between subtype C and B showed similar patterns, with the exception of some mutations, for example at positions 65, 103 and 106 (Figure 1.8). Few patients in this group received PIs, however there was a high frequency of potential primary (numbers in square boxes), secondary or accessory mutations among both treated and untreated patients. A number of other studies have confirmed the presence of such

baseline polymorphisms in subtype C in the protease regions (Cane, et al., 2001; Grossman, et al., 2001).

Mutations associated with the use of RTIs are similar to subtype B, with the exception of position 106 in RT where V106M has shown to be a subtype C-specific mutation in patients failing EFV (Figure 1.7). This is similar to the findings by Brenner et al., (2003), who demonstrated that a natural polymorphism occurred at this site in subtype C. Although this polymorphism coded for valine, the bases were GTG, not GTA, and occurred in approximately 94% of subtype C samples analyzed. This study demonstrated that under EFV pressure, valine changed to methionine (M) ATG in 3/6 subtype C samples (Brenner, et al., 2003). Morris et al., (2003) analyzed 15 HIV-1 subtype C samples failing EFV therapy and found 53% (8/15) harboured the V106M mutation. Moreover, one individual had only the V106M polymorphism in RT, implying it may represent a signature mutation for HIV-1 subtype C and a primary mutation that arises when subtype C is exposed to EFV. Thus, V106M may result in cross resistance to all NNRTIs (Brenner, et al., 2003). Interestingly in subtype C patients with no baseline mutations subjected to a single high dose of NVP to reduce MTCT, 5% had the V106M mutation and 3.5% the V106A mutation (Morris, et al., 2003).

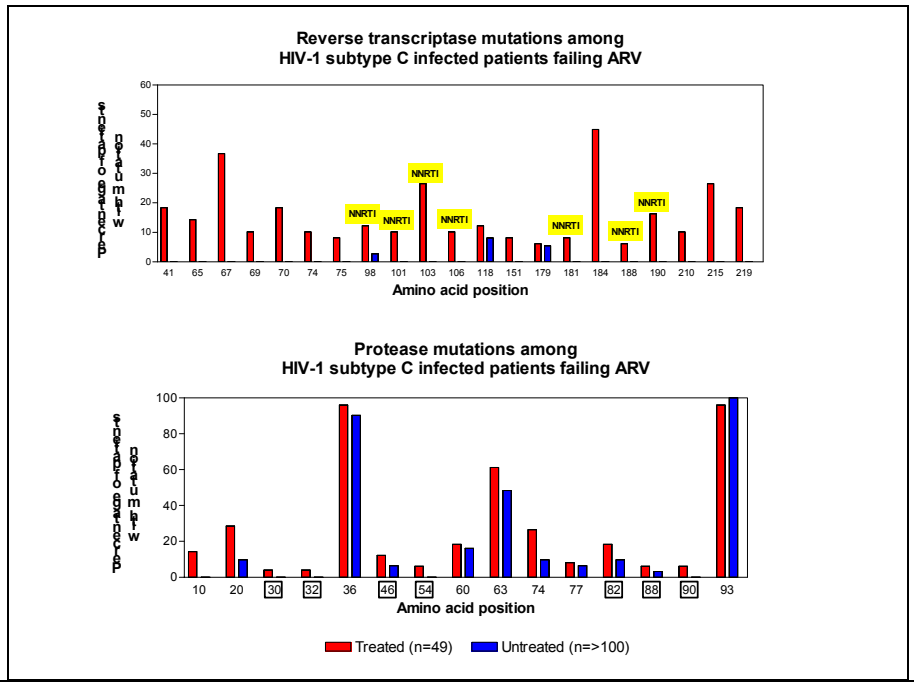


Figure 1.7: Point Mutations found in the a) PR and b) RT sequences of HIV-1 subtype C infected patients ARV naïve and treated in South Africa, (Courtesy L Morris).

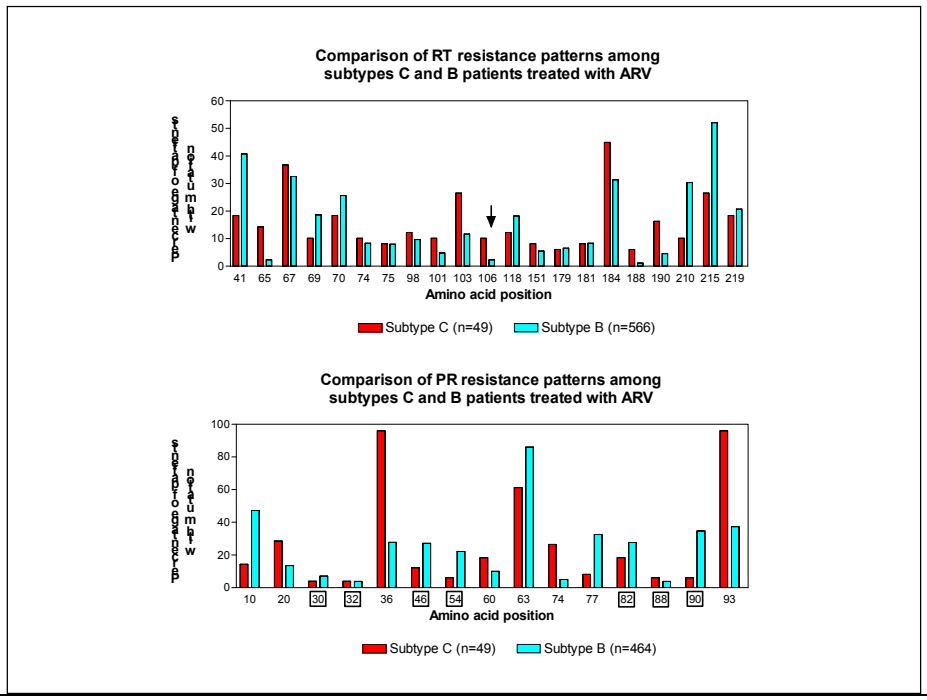


Figure 1.8: Mutations found in HIV-1 subtype C patients compared to mutations found in subtype B, (Courtesy L Morris).

1.9. **HIV drug resistance testing:**

HIV-1 drug resistance monitoring is becoming increasingly important as more patients access ARV treatment programs. The emergence of drug resistant viruses causes an increase in viral load and is indicative of treatment failure. Drug resistant profiles are important in the clinical management of patients and several studies have shown that physicians who have access to resistance data, are more likely to have patients that respond better to therapy (Baxter, et al., 2000, Cingolani, et al., 2002, Cohen, et al., 2002, Duval, et al., 2002). Furthermore, resistance testing is used to monitor transmission of drug resistant HIV for both RTIs and PIs (Yerly, et al., 1999), since infection with these strains will result in poor or no response to therapy in that individual. Transmission of drug resistant HIV is being monitored throughout the developed world. Studies on HIV from drug naïve individuals indicated that in Northern America 16% were resistant to NNRTIs (Boden, et al., 1999; Little, et al., 1999) and in the United Kingdom (UK) 11% were resistant to NRTIs, 3% to NNRTIs and 1% to PIs (UK collaborative group, 2001).

Guidelines for ARV drug resistance monitoring have been drawn up by the International AIDS society (IAS-USA), the United States (US) department of Health and Human Sciences and the Euro-Guidelines Group (EGG) (The Euro-Guidelines Group, 2001). The consensus is that resistance testing should be performed on drug naïve patients prior to therapy initiation if the background resistance in the overall infected population is greater than 5-10%. This will give information on baseline ARV resistance and aid the physician in drug choice. However, the EGG feels that ARV therapy should not be withheld if there is no drug resistance report available. Secondly, it should be performed on patients failing either first or second line regimens, to establish the reason for drug failure (Section 1.3)

and allow for correct drug prescription. Lastly, pregnant women should be screened prior to labour to ensure adequate prophylaxis to the neonate. In addition, infants that seroconvert, and are born to mothers that are failing therapy need to be tested (Hirsh, et al., 2000).

Patient plasma is the recommended source of sample material for ARV drug resistance testing. Plasma is thought to be more representative of the actively replicating viruses, unlike virus contained in the cellular component of the peripheral blood mononuclear cells (PBMCs). The abovementioned guidelines do not stipulate the use of a specific resistance assay, since both phenotypic and genotypic assays yield complementary information. The guidelines do however recommend that full sequencing methodologies are advantageous over detection of point mutations as they enable identification of novel mutations. However, it is thought that point mutation detection via assays such as Polymerase Chain reaction (PCR) may be more sensitive than sequencing based techniques. It is recommended that laboratories performing ARV resistance assays should be certified and have well-defined quality assurance standards.

Phenotypic assays are based on the generation of recombinant viruses, by complementation of an HIV backbone with RT and protease genes from patient samples. The replication rate of the recombinant virus is then examined under varying concentrations of ARVs. The production of recombinant virus is quantified to determine the levels of drug inhibition. However, discrepancies arise from the differences between drug metabolism in cell culture and in HIV infected individuals (Shao, et al., 2003). These assays give a direct measure of resistance but are very expensive. Moreover, these assays are not widely

performed as they require bio-safety level 3 laboratories, are time consuming, costly and pose significant risks to laboratory staff.

Genotypic assays (Population Based Sequencing) are currently the gold-standard and are based on test sequences being compared to a consensus sequence in a recognized database. Available population based sequencing assays include the FDA approved ViroSeq^{TM} HIV-1 Genotyping Version 2.6 (Applied Biosystems, Foster City, CA) which detects viral populations >40% and TruGene (Bayer Diagnostics). Genotypic assays are expensive, but are fast, safe and reproducible.

More sensitive genotyping methods include single-genome sequencing (SGS; Kearney, et al., 2003; Palmer, et al., 2003); and point mutation assays. SGS analyses a single copy of DNA, unlike conventional sequencing, which genotypes the entire quasi-species population. This assay uses RT-PCR to generate cDNA and the amplicon is subsequently serially diluted to a single copy. Fifteen to 46 single copies are then sequenced from each sample. The SGS method is able to detect drug resistant mutations in less then 10% of the population, which is below the limits of detection of population based genotyping (Kearney, et al., 2003), but very time consuming.

Various point mutation assays have been proposed as affordable alternatives to monitoring drug resistance by sequence analysis (Shafer, 2004) These type of assays identify specific drug related mutations, but are limited as they do not identify novel mutations; and need to be repeated to identify several different mutations. However, several studies have shown point mutation assays are more sensitive than population based sequencing (Shafer, 2004).

Point mutation assays described to date in this field include: a) Allele specific PCR assays; b) the Line Probe Assay (LiPa) and c) Oligonucleotide Ligation Assay (OLA). These methodologies are summarized below:

a) Allele specific PCR: this amplification method uses a nested PCR to determine if the mutation is present. If the mutation is present no amplicon will be generated (Larder and Boucher, 1993).

b) LiPa: hybridizes biotinylated PCR products to specific oligonucleotide probes immobilized on membrane strips. The probes are designed to detect either wild-type or mutant sequences. However, studies have shown that up to 25% of cases have discordant results compared to population based sequencing (Schmit, et al., 1998) and that several genotyped mutations were indeterminate (Re, et al., 2001).

c) OLA: detects known sequence variants in a standardised ELISA plate format. This assay amplifies RNA or DNA which serves as a template for allele-specific ligation reactions using three oligonucleotide probes (common, wild-type and mutant). Beck et al., (2002) have shown its effectiveness in the detection of point mutations in HIV-1 subtype B.

1.10. ARV Therapy in South Africa and plans for the national roll-out:

In 2003 the WHO wrote guidelines for 'scaling up ARV therapy in resource-limited settings' as part of the 3 by 5 initiative. These aim at increasing ARV access in resource poor settings, standardizing and simplifying regimens, and ensuring optimal drug regimens are used to reduce the chances of drug failure. South Africa's proposed national ARV roll-out is based on these guidelines (WHO, 2003). The roll-out is limited to district hospitals

and primary health care clinics, which have a clinician familiar with the ARV administration, and a comprehensive program for the prevention of MTCT. Moreover, these hospitals/clinics must have a regular ARV drug supply available. This program hopes to treat 50% of HIV infected people by 2010. There are currently two proposed drug regimens:

- The first drug regimen contains a combination of Stavudine, Lamivudine and Nevirapine or Efavirenz.
- The second regimen (for patients failing regimen one) contains Zidovudine, Didanosine and Kaletra (made up of Lopinavir and Ritonavir).

The RT inhibitors are the major component of the national drug roll-out program because of availability and cost. As mentioned previously, criteria for patient eligibility for enrolment on the program in South Africa includes a CD4 count < 200 cells/ml and/or stage 4 clinical diseases as described in the WHO clinical grading scheme (The WHO International collaborating Group for the study of the WHO staging system, 1993). Children must have a WHO paediatric stage 3 AIDS-defining illness or be symptomatic with a CD4 count of less than 15% or 20% of the average for their age group (First progress report, 2004). Because of the inevitable emergence of drug resistance to the prescribed regimens, drug resistance surveillance will need to be implemented on a national scale. Currently, the high costs associated with the available resistance assays will hinder their widespread use for individual patient monitoring and there is thus an urgent need for cheaper and faster alternatives.

This study aimed at evaluating and designing more affordable assays to detect the presence of point mutations in the HIV-1 RT gene, which confer resistance to the ARV drugs d4T, ddI and NVP/EFV. Proof of concept was achieved by evaluating OLA, designing a novel real time assay using a minor groove binding (MGB) modified probe for the K65R mutation, and a restriction fragment length polymorphism (RFLP) assay for V106M using the endonuclease *MaeIII*. Results were validated by comparison to the gold standard ViroSeq assay. These assays will allow for affordable ARV drug resistance testing in South Africa and further our knowledge on ARV drug resistance profiles in HIV-1 subtype C viruses circulating in our country and thereby allow for better treatment of HIV-1 infected individuals.

2. CHAPTER TWO: MATERIALS & METHODS

2.1. Sample Collection:

Three different starting sample materials were used throughout the course of this project: patient whole blood samples for DNA extraction; patient plasma for RNA extraction, and 1.8 kb PCR products amplified using the ViroSeq assay. The randomly chosen patient samples had viral loads ranging between 1040 to >750000 HIV-1 RNA copies/ml. Furthermore, only samples with known resistant profiles where used in the detection methods. Ethical clearance for use of patient sample materials was obtained through the human ethics committee of the University of the Witwatersrand using the research and development ethics clearance number: M00-01-07.

2.1.1. Patient Whole Blood

Nine patient whole bloods were obtained from the routine PCR diagnostic laboratory at the National Health Laboratory Service (NHLS) in Johannesburg, South Africa. DNA was extracted from all samples using the High Pure PCR Template Preparation Kit (Roche, Mannheim Germany), and used in PCR optimization (see Table 2.1). Whole blood from an HIV-1 seronegative individual was used as a negative control.

2.1.2. Patient Plasma

RNA from a total of 27 plasma samples (21 from NHLS; 6 from National Institute for Communicable Diseases (NICD) in South Africa) was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). RNA from a further 24 plasma samples (15 from National Institutes of Health Virology Quality Assurance (VQA) Laboratory and 9 from Contract Laboratory Services) were extracted using the methodologies

described in the ViroSeq™ HIV-1 Genotyping System Version 2.0 (Celera Diagnostics, CA). The RNA was used in various assays outlined in Table 2.1.

ViroSeq PCR Amplicons

Twenty eight previously amplified 1.8 kb PCR products encompassing the PR and RT genes (ViroSeq) were used to evaluate all methods (Table 2.1). A 1:10 dilution of each product was used. These amplicons were genotyped independently at the NICD (Table 2.2) and used for comparative purposes. Amplicons were selected on the basis of mutation profiles.

2.2. Nucleic Acid Extraction

2.2.1. DNA extraction

DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim Germany) according to manufacturer's instructions. Briefly, 200µl of whole blood was added to 200µl of binding buffer and 40µl of Proteinase K, and incubated at 72ºC for 10 minutes. Isopropanol (100µl) was then added and the entire mixture transferred to the High Pure Filter Tubes and centrifuged for 1 minute at 8000rpm. After centrifugation, 500µl of inhibitor removal buffer was added and centrifuged under the same conditions. To purify and remove all impurities from the nucleic acids 500µl wash buffer was added, and centrifuged under the same conditions. The wash step was repeated. To ensure removal of all the wash buffer the tube was further centrifuged at 13000rpm for 10 seconds. After each centrifugation step the flow through tube was discarded and replaced with a clean tube. Finally, the column was inserted into a 1.5ml tube and 200µl of a low salt elution buffer was added to release the nucleic acids from

the column and centrifuged at 8000rpm for 1 minute. The isolated DNA was stored at -70ºC until used in conventional polymerase chain reaction (PCR) (See Section 2.4.1).

Table 2.1: Outline of samples used in the research and development of ARV resistance assays

Sample Source	Number of Patients/ Samples	Sample Material	Extraction Method	Samples used in Assays					Laboratory Numbers
				PCR	OLA	MGB	RFLP	ViroSeq	
NHLS	8 HIV-1 positive	Whole blood	DNA (Roche)	X					NHWB1-14
	2 HIV-1 negative	1 Whole Blood and 1 Plasma	DNA (Roche) And RNA (Qiagen)	X					NEG1-2
	20 HIV-1 positive	Plasma	RNA (Qiagen)	X					NHP1-25
NIH	15 HIV-1 positive	Plasma	RNA (Applied Biosystems)/ ViroSeq	X	X			X	VQA004G01-50 VQA005g01-05 VQA006g-1-5
NICD	6	Plasma	RNA (Qiagen)	X	X	X	X	X	DR001-0102
	28	ViroSeq PCR product		X	X	X	X	X	DR001-0102
CLS	9 HIV-1 positive	Plasma	RNA (Applied Biosystems)/ ViroSeq	X				X	1-9

.

Table 2.2: ARV drug resistance profiles for 28 ViroSeq amplicons (see Table 2.1).

Name	Mutations in RT	Current Drug Regimen	Subtype
DR006	K103N; Y181C	N/A	C
DR010	M41L; E44D; D67N; T69D; A98G; V118I; M184I; Y188L; G190A; L210W; T215Y	d4T; 3TC; EFV	C
DR012	K103N; Y181C	EFV; IDV; RTV	B
DR016	M41L; E44D; D67N; T69D; A98G; V118I; M184I; L210W; T215Y	Trizavir, Combivir,NFV	C
DR017	M41L; D67N; T69D; K70R; V75M; L210W; T215F; K219Q	AZT, ddI	
DR019	M41L; D67N; T69E_SS; K101E; G190A; L210W; T215Y	AZT, ddI, EFV	C
DR021	K70R; F77L; V118I; Y181C; M184V	Combivir, NVP	B
DR028	A98G; K103N; M184V; T215Y; M23OL	AZT, 3TC, EFV	C
DR029	D67N; K70R; K103N; G190A; K219Q	Combivir, NFV	C
DR033	L74V	ddI, d4T, HU	C
DR037	D67N; K70R; L74V; M184V; K219E	ddI, ABC, NFV	C
DR041	M41L; E44D; L74V; A98G; Y181C; M184V; L210W; T215Y; K219K/N; M230L	AZT, 3TC, NVP	C
DR044	K65R; L74V	d4T, ddI, RTV	C
DR047	K65R; D67N; K103N; V106M; F116Y; Q151M	ddI, d4T, EFV	C
DR048	M41L;D67N;K70R;V75I;F116Y;Q151M;Y181C;G190S;T215Y;K219E	d4T, ddI, EFV, NFV	B
DR050	K103N; V106M; M184V	Combivir, EFV	C
DR051	M41L; K103N; M184V; P225HP	AZT, 3TC, IDV	C
DR056	T69N; L74V; L100I; K101P/Q/T; K103N	d4T, ddI, EFV	C
DR059	A62V; K65R; V75I; F77L; A98S; F116Y; Q151M	d4T, ddI	C
DR064	Y181C; M184V; G190A	3TC, NVP, ZDV	C
DR068	A62V; K65R; V75I; F77L; F116Y; Q151M; M184V	AZT, 3TC, RTV	C
DR069	K65R; D67N; V106M; Y188C	d4T, ddI, EFV	C
DR084	K103R; V179D; Y188L; G190A	ddI, d4T, EFV	C
DR092	M41L;D67N; T69D; K70R; V106M; Y188C; G190A;T215C; K219Q	d4T, EFV, LPV	C
DR094	K103N; M184V; P225H	AZT, 3TC, EFV	C
DR095	M41L; L74V; K103N; V106M; T215Y	Combivir, d4T, ddI, EFV	C
DR098	D67N; K70R; K103N; V106M	d4T, ddI, EFV	C
DR0102	A62V; T69S; V75I; F77L; F116Y; Q151M	d4T, ddI, RTV	C

2.2.2. RNA extraction

2.2.2.1. RNA extracted from NHLS and NICD plasma samples

Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany). Firstly, 140µl of plasma was centrifuged for 1 hour at 23000xg at 4ºC to pellet the

HIV-1 viral particles, and then 560µl of AVL buffer containing carrier RNA added. The buffer and plasma were incubated for 10 minutes at room temperature. To remove unwanted proteins and cellular debris, 560µl of 100% ethanol was added and centrifuged at 6000xg for 1minute and the filtrate discarded. Contaminants were removed by two 500µl washes using buffers AW1 and AW2, and centrifuged for 1minute at 6000xg and 3minutes at 20000xg, respectively. The captured RNA was eluted in 60µl AVE buffer by centrifuging for 1 minute at 6000xg. Eluted RNA was stored at -70ºC until used in RT-PCR methods (Section 2.4.2).

RNA yield was determined using the RiboGreen kit (Roche, Germany). Working Solutions of the RiboGreen (1:200) and RNA standard (1:50) was achieved by diluting in 1xTE. The RNA working stock was serially diluted from 1000ng; 500ng; 100ng; 20ng and 0ng, and added to the RiboGreen working Solution in a ratio of 1:1. The Optical densities (OD) were obtained on the LightCycler I (Roche Diagnostics, Germany) and used to plot a standard curve. Isolated RNA was diluted (1:10 and 1:50) and added to RiboGreen working solution in a 1:1 ratio. The OD of each sample was read on the LightCycler I and the RNA yield determined from the standard curve.

2.2.2.2. Plasma samples from the National Institutes of Health Virology Quality Assurance Laboratory

These plasma samples are part of the Virology Quality Assurance (VQA) program. These samples were isolated using the RNA extraction kit provided in the ViroSeq™ HIV-1 Genotyping System Version 2.0, Pack 1 (Celera Diagnostics, Alameda, CA, USA). Briefly,

500µl of plasma was centrifuged for 1 hour at 23000xg at 4ºC, to pellet the HIV-1 viral particles. The viral pellet was resuspended in 600µl lysis buffer to lyse the viral particles and release the HIV-1 RNA. The RNA was purified by two 600µl washes using isopropanol and cold 70% ethanol, respectively and the supernatant discarded. The RNA pellet was eluted in 50µl elution buffer containing RNases to ensure intact RNA was obtained. The eluted RNA was stored at -70ºC, until amplified.

2.3. ViroSeq™ HIV-1 Genotyping System Version 2.0: Gold Standard Sequencing assay

2.3.1. Amplification

The isolated viral RNA (Section 2.2.2.2) was used to generate HIV-1 cDNA (Figure 2.1). In this process the cDNA was generated using 10µl of reaction mix (Murine Moloney (MuLV) reverse transcriptase, RNase Inhibitor and RT mix containing a specific reverse primer) and 10µl of RNA. The 20µl of synthesized cDNA was used as a PCR template. This amplification process used 30µl of amplification mixture (Ampligold Taq, dUTP/UNG and a PCR mix containing HIV-1 specific primers). The PCR reaction was used to generate an approximately 1.8kb fragment including the coding sequence for HIV-1 protease (the entire protein, amino acids 1-99) and part of the HIV-1 reverse transcriptase (amino acids 1-324). To remove excess primers and buffers the 50µl of amplicon was purified using microconcentrators (Celera Diagnostics, CA). The PCR product was analyzed and quantified on a 2% agarose gel (Appendix A.2; Figure 2.1). The size and concentration of the DNA

fragment was identified by comparing it to a DNA Mass Ladder (Celera, Diagnostics, Alameda, CA).

2.3.2. Dideoxy Sequencing

The samples were diluted according to their concentration and dideoxy (chain termination) sequencing performed, using the Sequencing Module of the ViroSeq HIV-1 Genotyping Kit. Twelve microlitres of primer mix were added to 8µl of appropriately diluted amplicon. Seven sequencing reactions were set up for each sample, and included 4 sense (A, B, C, D) and 3 anti-sense (F, G, H; Figure 2.1) primers. The cycling conditions were: 96ºC for 10 minutes; 50ºC for 5 seconds, 60ºC for 4 minutes for 25 cycles (GeneAmp® PCR System 2700, Applied Biosystems, Singapore). After cycle sequencing unincorporated ddNTPs were removed by adding 80µl of 80% isopropanol to each sample. Incubation for 15 minutes at room temperature was followed by a centrifugation step to pellet the sequence amplicon (45 minutes at 4000xg). The plate was immediately inverted to remove the supernatant by centrifugation for 1 minute at 700xg. To the dried pellet 20µl of Hi-Di™ Formamide (Applied Biosystems, Warrington, UK) was added.

2.3.3. Analyses of Generated Sequences

The DNA fragments now labelled with four different fluorescent dyes (one for each base) were loaded onto the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem, HITACHI, Foster City, USA). The sequence information was processed by the ABI PRISM ® 3100 Data Collection Software version 1.1 (Applied Biosystem, USA; Figure 2.1), and assembled and analyzed on the ViroSeq™ HIV-1 Genotyping System Software Version 2.6 (Applied

Biosystems, Foster City, CA, USA). Manual reviewing and editing was performed, while the generated sequence was compared to a reference sequence. Once manual editing was completed the following files were saved: 1) FASTA file, containing the actual sequence; 2) GT file, containing a mutation list and 3) a report showing the mutations and drugs no longer effective for the patient. The FASTA file was submitted to the HIV Drug resistance database (2005) for subtyping. The ViroSeq Assay is able to detect a viral population >40% (ViroSeq™ HIV-1 Genotyping System version 2.0 Operators Manual, 2003).

To ensure that the ViroSeq assay was efficiently working in the genotyping laboratory, in Johannesburg, SA, appropriate training was undertaken at the Genotyping Laboratory at John Hopkins, Baltimore, USA. This laboratory was responsible for the validation process carried out to get FDA approval of this assay.

Samples from the 004g01-04, 005g01-05 and 006g01-05 NIH VQA proficiency panels were sequenced in our laboratories and independently compared to sequences generated in other laboratories participating in the VQA program worldwide. Furthermore the amplicons from the 004g01-05 and 005g01-05 samples were used in OLA.

ViroSeq HIV-1 Genotyping rocess Summary Diagram

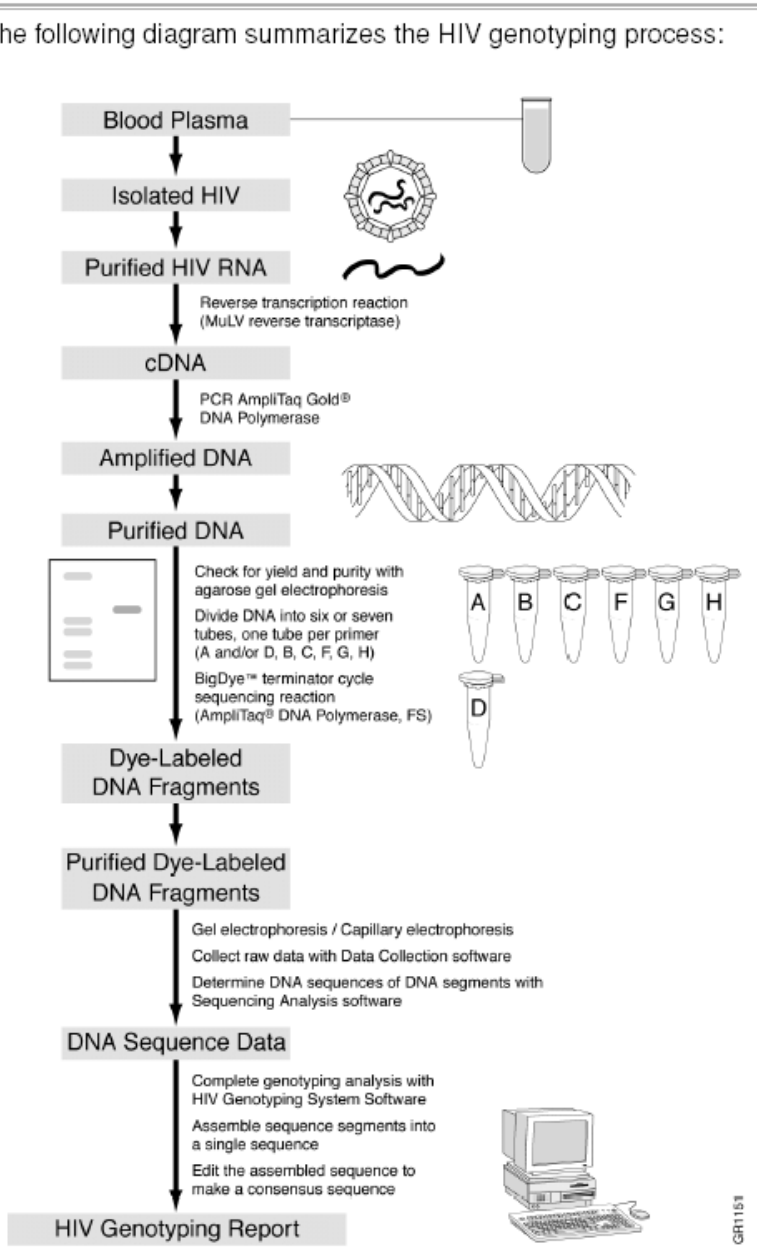


Figure 2.1: Step-by-step procedure followed when performing the ViroSeq Assay (ViroSeq Package Insert, 2003).

2.4. Conventional PCR:

Conventional PCR reactions were performed on isolated DNA and RNA (Section 2.2). The nucleic acids were amplified using different primer sets: a) those supplied in the OLA kit, (NIH AIDS Research and Reference Reagent Program cat number: 7644 and 7645), b) previously published primers (Larder & Boucher, 1993) and c) ST1 and ST2 (Table 2.3). ST1 and ST2 were designed from a comparative study of 78 HIV-1 subtype C reverse transcriptase sequences obtained from ARV drug naïve women (Pillay et al. 2002). These sequences were aligned using the GeneDoc program (Karl Nicholas, 2003), and conserved regions in the RT gene identified. Primers with similar melting temperatures were designed in this conserved region and checked on Primer3 Input, version 2.0 (Rozen & Skaletsky, 2000).

Table 2.3: Sequences of primers used for amplification of nucleic acids (Beck and Frankel, 2002; Larder and Boucher, 1993).

	Name	Direction	Sequence	Size
OLA first round (Beck and Frankel, 2002)	PRA	Forward	5'-CCT AGG AAA AAG GGC TGT TGG AAA TGT GG-3'	1197 base pairs (gag to codon 238 of RT)
	RTA	Reverse	5'-AAC TTC TGT ATG TCA TTG ACA GTC CA-3'	
OLA second round (Beck and Frankel, 2002)	PRB	Forward	5'-ACT GAG AGA CAG GCT AAT TTT TTA GGG A-3'	1060 base pairs (gag to codon 237 of RT)
	RTB	Reverse	5'-CAT TTA TCA GGA TGG AGT TCA TA-3'	
Primers (Larder and Boucher, 1993).	A-(35)	Forward	5'-TTG GTT GCA CTT TAA ATT TTC CCA TTA GTC CTA TT-3'	805 base pairs of the PR and RT enzyme
	NE-1(35)	Reverse	5'-CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT-3'	
Subtype C primers	ST-1	Forward	3'-AAA ATT GGG CCT GAA AAT CC-5'	543 base pairs of the RT enzyme
	ST-2	Reverse	3'-ATC CAA AGA AAT GGG GGT TC-5'	

2.4.1. Amplification of DNA:

The isolated DNA was amplified either by a single amplification step, or a nested PCR (Figure 2.2).

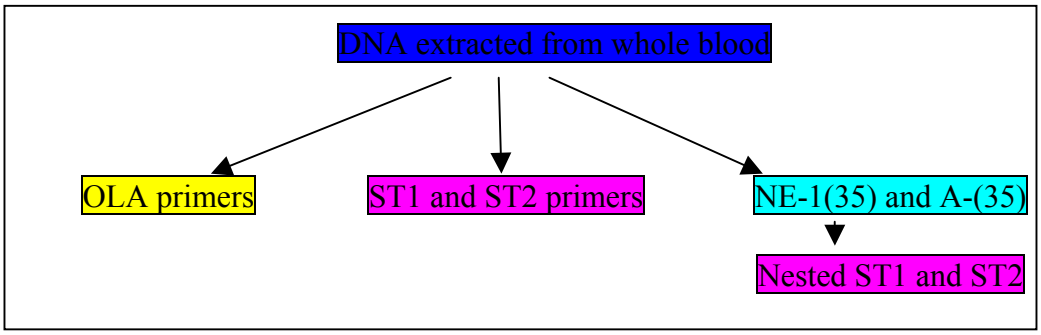


Figure 2.2: Overview of primer sets used in three different reactions to amplify PCR product from DNA.

2.4.1.1. PCR amplification using OLA primers

DNA extracted from whole blood (Table 2.1) and the DNA control provided in the OLA kit were amplified according to instructions in the OLA protocol provided in the kit (Beck et al, 2002). This amplification involved a nested PCR reaction. Briefly, DNA was amplified using first round primers PRA and RTA, and subsequently second round primers PRB and RTB (Table 2.3). A total volume of 50µl was used for both the first and second round reactions. For both reactions, 1x PCR buffer (Fermentas, Lithuania), 2.5mM $MgCl_2$ (Fermentas, Lithuania), 0.2mM dNTPs (AB gene, Surrey, United Kingdom), 2.5U Taq (Fermentas, Lithuania) and 0.4pmol of each primer was used. Moreover, 2µl of DNA and first round product was used for each reaction.

The cycling conditions consisted of an initial denaturation step of 95°C for 7 minutes, followed by 35 cycles of 95°C for 20 seconds, 55°C (first round) and 52ºC (second round) for 20 seconds, and 72°C for 1 minute, and a final elongation step of 72°C for 7 minutes.

2.4.1.2. PCR amplification using primers A(35) and NE-1(35) and ST1 and ST2

The first round PCR reaction was performed using the primers A-(35) and NE-1(35) (Larder and Boucher, 1998), and a nested PCR performed using primers ST1 and ST2. The first round reaction was carried out in a 100µl reaction volume, containing 2µl of DNA, 0.2mM dNTPs (AB gene, Surrey, United Kingdom), 0.4pmol of primers A-(35) and NE-1(35), 2.5mM $MgCl_2$, 1xPCR buffer and 0.05U/µl Taq DNA polymerase (Fermentas, Lithuania). The cycling conditions consisted of an initial denaturation step of 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, and a final elongation step of 72°C for 7 minutes. The nested PCR reaction was carried out in a 40µl reaction volume, containing 4µl of first round PCR product, 2.5mM dNTP's (AB gene, Surrey, United Kingdom), 2pmol of primers ST1 and ST2, 1.5mM $MgCl_2$ (Fermentas, Lithuania), 1xPCR buffer and 0.05U/µl Taq DNA polymerase (Fermentas, Lithuania). The optimized cycling conditions consisted of an initial denaturation step of 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, and a final elongation step of 72°C for 7 minutes.

2.4.1.3. PCR Amplification using primers ST1 and ST2

This single round amplification reaction was performed as described in section 2.4.1.2 using 20µl of DNA as the starting material.

2.4.2. *PCR Amplification of viral RNA:*

RNA amplification was performed as a two-step and a one-step RT-PCR, using the OLA primers, A-(35) and NE-1(35) and ST1 and ST2 (Figure 2.3).

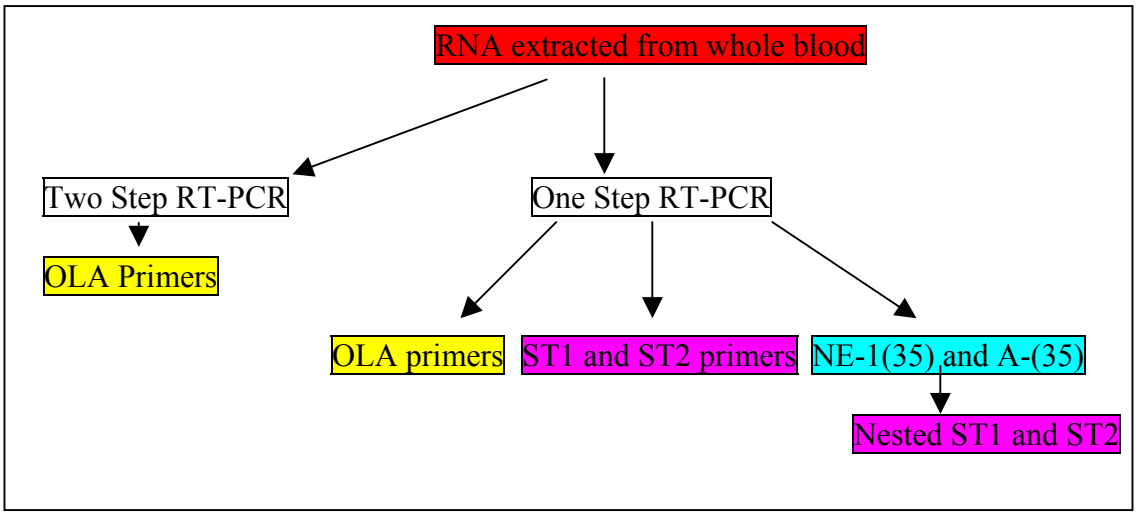


Figure 2.3: Overview of primer sets used in four different reactions to amplify PCR product from RNA.

2.4.2.1. Two step RT-PCR using OLA primers

The reverse primer RTA was used to synthesize the cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™, Carlsbad, CA). Briefly, 8µl of RNA and 0.5mM dNTPs were added together and incubated for 5 minutes at 65ºC. The samples were snap cooled for one minute and 1x RT buffer, 5mM $MgCl_2$, 0.01 M DTT and 1U RNase OUT

added in a total volume of 20µl. This mixture was incubated for 2 minutes at 42ºC and then 1U of SuperScript II RT was added. Once the cDNA was transcribed, the OLA protocol was followed to synthesize amplicon as describe in section 4.1.1 above.

2.4.2.2. One-step RT-PCR using the OLA primers

The extracted RNA was amplified using the Titan One Tube RT-PCR System (Roche, Penzberg, Germany) followed by a nested PCR reaction. Both PCR reactions performed used the primers supplied in the OLA kits. This one-step reaction was carried out in a total reaction volume of 50µl, containing 10µl of RNA and 0.4pmol of primer PRA and RTA, 0.2mM dNTPs, 5mM DTT and 5Units RNase Inhibitor, 5x RT-PCR with a final $MgCl_2$ concentration of 1.5mM and 1µl of enzyme mix. The amplification cycle included an annealing temperature of 50^{o}C for the non-specific cycling step (10 cycles) and 55^{o}C for the specific cycling step (40 Cycles). The nested PCR was performed on the first round PCR product as described in section 2.4.1.1.

2.4.2.3. One-Step RT-PCR using primers A-(35) & NE-1(35), and ST1 & ST2

The RNA was amplified using a one-step PCR followed by a nested PCR reaction. The one-step was performed using the primers A-(35) and NE-1(35) (Larder and Boucher, 1993), and a nested PCR performed using primers ST1 and ST2. This one-step reaction was carried out as described by the Titan One-Step kit protocol, using a total reaction volume of 50µl, containing 10ul of RNA and 0.4pmol of primers A-(35) and NE-1(35), 0.2mM dNTPs, 5mM DTT and 5Units RNase Inhibitor, 5x RT-PCR with a final $MgCl_2$ concentration of 1.5mM and 1µl of

enzyme mix. The amplification cycles were as described in section 2.4.2.2. The nested PCR using primer ST1 and ST2 was performed as described in section 2.4.1.3.

2.4.2.4. One-step RT-PCR using primer ST1 & ST2

To amplify RNA using the ST1 and ST2 primers, the Titan One-Step RT-PCR kit was used. This one-step reaction was carried out as described in the kit protocol, using a total reaction volume of 50µl, containing 10µl of RNA and 0.4pmol of primer ST1 and ST2, 0.2mM dNTPs, 5mM DTT and 5Units RNase Inhibitor, 5x RT-PCR with a final $MgCl_2$ concentration of 1.5mM and 1µl of enzyme mix. Cycle conditions were as described in section 2.4.2.2.

2.4.3. Amplification of ViroSeq amplicon:

Amplicon was amplified, using the OLA primers, A-(35) and NE-1(35) and ST1 and ST2 (Figure 2.4).

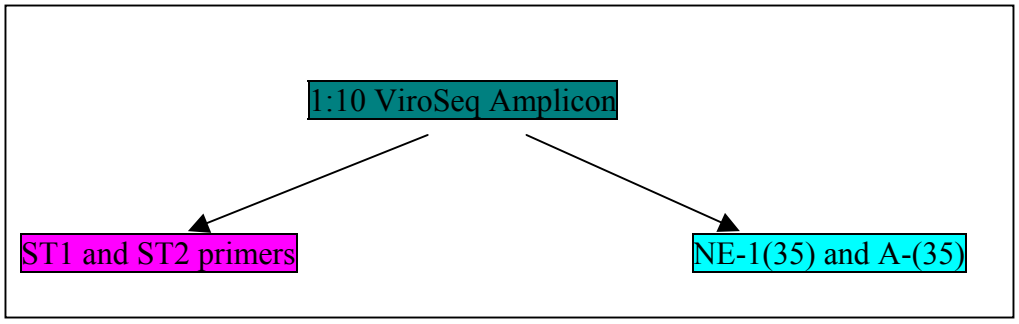


Figure 2.4: Overview of primer sets used in two different reactions to amplify PCR product from ViroSeq Amplicon.

2.4.3.1. 1:10 ViroSeq product using primers ST1 & ST2:

The previously genotyped PCR product obtained from the ViroSeq RT-PCR (Table 2.1) was used as starting material for the nested PCR reaction, using the ST1 and ST2 primers. The nested PCR reaction was carried out as described in section 2.4.1.3 and the product was subsequently used for the detection methods: OLA and RFLP analysis.

2.4.3.2. ViroSeq PCR product using priners A-(35) and NE-1(35)

The 1:10 diluted ViroSeq PCR product obtained was amplified using primers A-(35) and NE-1(35), and the procedure described in 2.4.1.2 followed. This product was eluted and used in the MGB detection (Section 2.6.3).

2.4.4. Controls:

Plasmid Controls (Table 2.4) were amplified, using the OLA primers and ST1 and ST2 (Figure 2.5).

Table 2.4: Control plasmids supplied by the OLA kit

Plasmid	**Reference Genotype**
p8E5	Wild-type
pNVP	K103N and Y181C
p65R	K65R; D123E; D177E; T200I and I202V
pMDRI	T215Y; M41L; L74V; V106A
p151M	Q151M; F77L; V118I; M184V and F214L

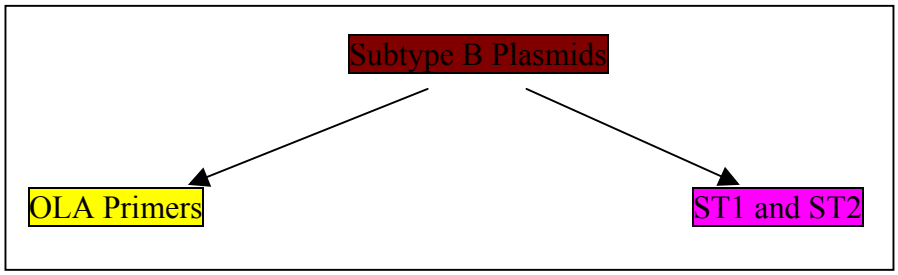


Figure 2.5: Overview of primer sets used in two different reactions to amplify PCR product from plasmids.

2.4.4.1. PCR amplification using OLA primers

Plasmids provided in the OLA kit (Table 2.4) were amplified according to instructions in the OLA protocol provided in the kit (Beck and Frankel, 2002). Plasmids were amplified using primers (5'-CTC CCC CTC AGA AGC AGG AGC CGA TAG ACA AGG AAC T-3') and RT3 (5'-TAT CAG GAT GGA GTT CAT AAC-3'). A total volume of 50µl was used with 1x PCR buffer (Fermentas, Lithuania), 2.5mM $MgCl_2$ (Fermentas, Lithuania), 0.2mM dNTPs (AB gene, Surrey, United Kingdom), 2.5U Taq (Fermentas, Lithuania) and 0.4pmol of each primer. 2µl of plasmid was used in the reaction. The cycling conditions consisted of an initial denaturation step of 95^{o}C for 7 minutes, followed by 35 cycles of 95^{o}C for 20 seconds, 55^{o}C for 20 seconds and 72^{o}C for 1 minute, and a final elongation step of 72^{o}C for 7 minutes.

2.4.4.2. 1:10 ViroSeq product using primers ST1 & ST2:

Two microliters of plasmids was used as starting material for the nested PCR reaction, using the ST1 and ST2 primers. The nested PCR reaction was carried out as described in section 2.4.1.3 and the product was subsequently used all detection methods evaluated.

All PCR products obtained were visualized on a 2% agarose gel (Appendix A.2) following electrophoresis and ethidium bromide staining to ensure successful amplification.

2.5. Gel Elution

Successfully amplified samples, plasmids and positive controls were eluted from the gel using the GFX PCR DNA and gel band purification kit (Amersham Biosciences, England). Briefly, the amplified product's band was cut out from the gel and weighed. A proportional amount of capturing buffer was added. This buffer contains a chaotropic agent which during a 15 minute incubation at 60ºC denatures proteins and dissolves the agarose. The sample was transferred to the GFX column and incubated for 1 minute and then centrifuged for 30 seconds at full speed, and the flow through discarded. The captured DNA strands were washed with 500µl wash buffer and centrifuged for 1 minute at full speed. The DNA was eluted into a clean tube using 50µl of elution buffer and a centrifugation step of 1 minute at full speed. Eluted PCR product was used in the MGB assay (Section 2.6.3).

2.6. Evaluation of Alternative Methodologies for ARV drug resistance detection

Three different methods for detection of ARV drug resistance point mutations were evaluated (RFLP, MGB and OLA). All three assays used the same ViroSeq PCR products amplified by the ST1 and ST2 primers (Section 2.4.3.1).

2.6.1. Oligonucleotide Ligation Assay (OLA)

The OLA assay was evaluated for its efficiency in detecting ARV drug resistant HIV-1 subtype C isolates, since it was originally designed for use in HIV-1 subtype B infected populations. Several mutations were selected for evaluation with this kit based on the treatment regimens implemented in the SA national rollout program (Section 1.10), namely, ddI, d4T and EFV/NVP. The following kits were selected: 1) the **NRTI OLA reagent kit** containing 6 primers, 13 codon-specific oligonucleotides and a control genomic DNA. The mutation K65R was evaluated with this assay, 2) the **NNRTI OLA reagent kit** containing 6 primers, 6 codon specific oligonucleotides and control genomic DNA. From this kit, the mutations K103N and Y181C were studied.

Test samples and controls provided were tested in duplicate during the course of each assay. Briefly, 2µl of PCR product, 10ul of 0.1% Triton X-100 (Sigma, germay) and 10µl of ligation mix (240µl 10x ligase buffer-Appendix A.3; 240µl 10mM NAD (Roche, USA); 30µl 1M KCl (MERCK, South Africa); 690µl 0.1% Trition X-100; 8µl wild-type oligonucleotide, mutant oligonucleotide and common oligonucleotide; 4µl Ampligase DNA ligase, Epicentre) were added to each well of a 96 well plate. The plate was placed on the thermocycler for 10 cycles of 93ºC for 30 seconds and 37ºC for 4 minutes to ensure ligation of the codon-specific probes to the template.

The ligation was stopped by adding 10µl of 0.1M EDTA/0.1% Triton X-100. The ligated product was captured on a streptavidin- coated plate (StreptaWell, Roche, Mannheim,

Germany) and incubated at room temperature for one hour to ensure binding. The plate was washed with 1xNaOH (Appendix A.5) and 1x Tris (Appendix A.6) wash buffer to remove unbound product. Forty microliters of antibody solution (5ul Anti-digoxigenin-POD, Roche, Mannheim, Germany; 5ul anti-fluorescein-AP Roche, Mannheim, Germany; 5ml Bovine Serum Albumin (BSA) Blocking Solution-Appendix A.4) was added and left to bind for 30 minutes. The plate was washed six times with 1xTris wash buffer to remove unbound antibodies.

The mutant genotype was detected by adding 25µl of GIBCOBRI® amplifier for 10min at room temperature and adding 25µl of GIBCORBI® substrate (GIBCOBRI®, Paisley, Scotland) and waiting until the colour changed to magenta. The Optical Density (OD) reading was taken on the ELx800 spectrophotometer (Bio-Tek Instruments, USA) at 490nm. The wild-type genotype was then detected once the GIBCORBI substrate and amplifier was removed by six 1xTris washes. Fifty microliters of 3,3',5,5'-Tetramethylbenzidine (TMB, Roche, Indianapolis, IN, USA) was added to each well and incubated for 10 minutes at room temperature for a blue colour to appear. The blue colour was then changed to yellow by adding 50µl of 0.3M H_2SO_4 (MERCK, South Africa) and the OD reading taken at 450nm (Beck and Frankel, 2002). All OD readings were subtracted from an average of 4 blanks run with each assay. The OD readings obtained were plotted on a scatter plot (mutation versus wild-type) and the cut-off for both mutant and wild-type determined using the mean OD for either wild-type/mutant plasmid ± 2.5SD (Beck, et al., 2002). To control for variation

between assays both wild-type and mutant plasmid and DNA controls for each codon tested was included in every run.

2.6.2. Restriction Fragment Length Polymorphism (RFLP) Analysis:

This assay is based on an endonuclease enzyme which cleaves double-stranded DNA at a specific site in a defined manner (restriction site). As an example, this was exploited to establish RFLP to detect the V106 mutants conferring resistance to all NNRTIs. To determine which enzymes will best cut at the position 106 mutation site, the Webcutter version 2.0 (Heiman, 1997) was used. The nucleotide sequence of PCR products generated by primers ST1 and ST2 was added into the program, with and without relevant mutations and analyzed for an appropriate restriction enzyme site. This resulted in the discovery of a unique restriction enzyme site in the V106M/A mutation. The restriction enzyme *MaeIII* recognizes the sequence /GTNAC. The degenerate base (N) in the enzyme's recognition sequence allows for both HIV-1 subtype B and C sequences to be restricted, sequence GTA (subtype B) and GTG (subtype C). When either mutation A (ATG) or M (GCA) is present, *MaeIII* does not recognize codon 106 as a restriction site. A similar approach was undertaken to detect the M184V mutation using the restriction enzyme *Nla*III (Stevens, et al., 1999).

Figure 2.6 represents the fragment sizes which should be generated after the wild-type or mutant PCR fragment have been restricted. If no mutation is present at codon 106 and the amino acid is valine, irrespective of subtype polymorphism, the PCR product will be restricted into 2 fragment sizes of 408 and 135 base pairs. However if a mutation occurs at codon 106,

be it A/M/I, the restriction enzyme site will be absent and the PCR product will not be digested (543 base pairs).

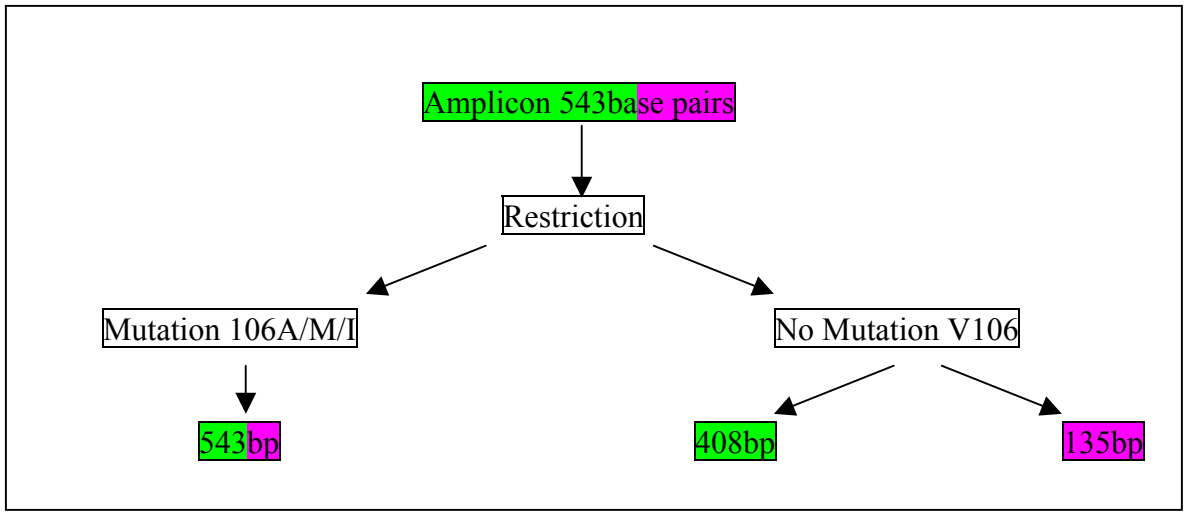


Figure 2.6: Represents the fragment sizes which are produced after the 543 PCR fragment has been restricted. If there is a mutation present at codon 106, no restriction will occur, however when no mutation is present at codon 106, the PCR product will be restricted into 2 fragments 408 and 135 base pairs.

For this assay, 4 HIV-1 subtype B recombinant plasmids and 1 positive DNA control (OLA kit), 1 negative HIV-1 DNA sample and 12 of the 28 HIV-1 subtype C previously amplified samples were analysed (Table 2.1). All samples were first PCR amplified in a nested reaction using the ST1 and ST2 primers (Section 2.4.3.1). Prior to restriction the PCR product was electrophoresed on a 2% agarose gel (Appendix A.2) to check for the presence of the correct PCR product (543 base pairs).

The 543 base pair products were restricted in a total volume of 25µl. The restriction enzyme digest reaction was comprised of 22.5µl of PCR product, 2ul of 10 x restriction buffer, and 1ul (1unit) of the *MaeIII* enzyme (Roche, Germany). This reaction mixture was incubated at 55^{o}C

for 1 hour to completely cleave the DNA. After one hour, the reaction was inactivated using 2µl of bromophenol blue (Appendix A.7). The restricted fragments were analyzed on a 3% agarose gel (Appendix A.8). For every reaction a positive and negative control was included.

2.6.3. Minor Groove Binding Assay

The potential of an in-house point mutation assay using MGB modified probes on the LightCycler II (Roche Diagnostics, Germany) was explored. The LightCycler II was selected due to speed of the reaction, furthermore this machine has the ability to multiplex fluorescent probes. The two fluorescent probes (wild-type and mutant) used, were labeled with two different dyes, FAM and VIC, respectively, thereby requiring a multi-channel machine such as the LightCycler II. The MGB complex is a naturally occurring antibiotic which binds to a duplex DNA strand, via the phosphate sugar backbone and is stabilized by the hydrogen bonds and hydrophobic interactions. This stabilization allows for an increase in melting temperature of about 15ºC and therefore a decrease in probe length to 12 bases (Afonina, et al. 2002). The short length of the MGB modified probe allows for increased specificity resulting in better mismatch discrimination. The MGB probe was designed to detect the K65R primary mutation which is associated with drug resistance of most NRTIs.

Eluted PCR product obtained in section 2.5 was diluted to 1:50 concentration and amplified using the LightCycler FastStart DNA Master^{PLUS} Hybridisation Probes Kit (Roche, Penzberg Germany). Two microliters product was added to 4ul of master mix, 0.4pmol of the forward (5'-TGG GCC TGA AAA TCC ATA TAA CAC-3') and reverse (5'-GGT ATT CCT AAT

TGA ACC TCC CAA A-3') primer, 0.4pmol of the FAM probe (6-fam-ATT TGC CAT AAA AAA GA-MGB) and the optimized 0.6pmol of the VIC probe (vic-TAT TTG CYA TAA AAA GGA A-MGB; Applied Biosystems, Cheshire, United Kingdom) and added to water to a total volume of 20µl. The amplification was performed on the LightCycler II and the following optimized program followed: Pre-incubation for 10 minutes at 95ºC, 35 amplification cycles of 95ºC for 10 seconds, 62ºC for 20 seconds, 72ºC for 10 seconds, with the single quantification at the elongation step. Finally samples were cooled to 40ºC for 30 seconds. The amplicons were analyzed using the qualitative detection option.

3. CHAPTER THREE: RESULTS

3.1. Extracted Viral RNA Quantification

To ensure that a sufficient amount of viral RNA was extracted for RT-PCR, 22 samples were quantified using RiboGreen. A standard curve was generated from a series of dilutions and a line of regression plotted from the optical densities (OD) and known concentrations. This curve was used to determine the viral RNA concentrations of each sample (Figure 3.1). The standard curve generated has a slope of 0.9985 indicating the accuracy of the dilution. The closer the slope (line of regression) is to 1, the more accurate the dilution series. From the line of regression, an equation was generated and used with the sample OD readings to determine their concentrations (Table 3.1). The sample concentrations indicated that a sufficient amount of viral RNA was extracted from each sample.

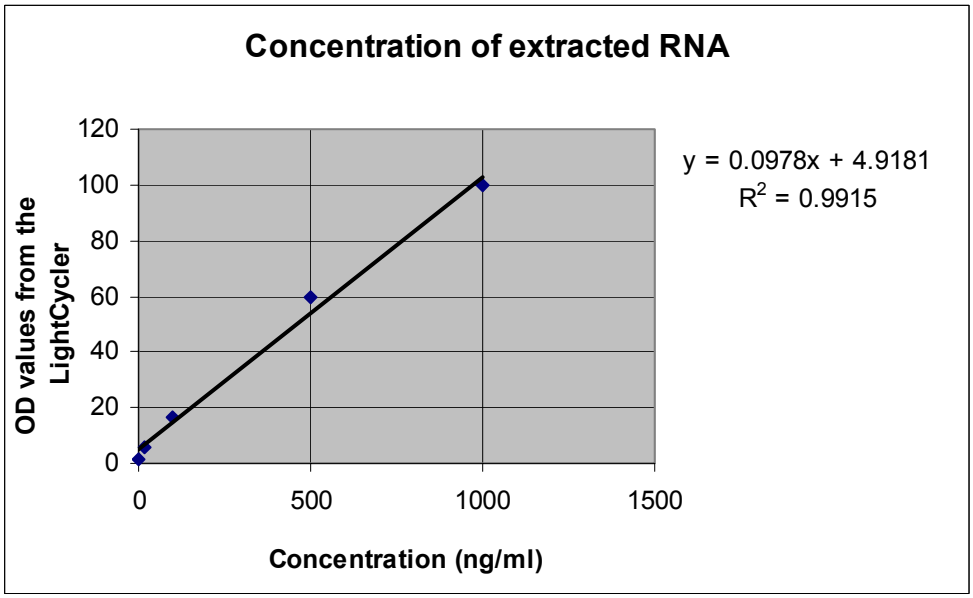


Figure 3.1: An example of a standard curve used to quantify extracted viral RNA.

Table 3.1: Concentrations of extracted viral RNA samples determined from Ribogreen standard curve.

Sample Name	ng/ml of RNA
NHP1	23.2
NHP2	27.3
NHP3	46.6
NHP4	20.1
DR016	29.30
DR047	8.16
DR094	12.81
DR095	73.17
DR037	70.9
DR050	26.04
NHP5	81.49
NHP6	11.10
NHP7	10.60
NHP8	17.17
NHP9	46.93
NHP10	92.79
NHP11	63.49
NHP12	18.83
NHP13	20.6
NHP14	39.63
NHP15	43.91
NHP16	43.69

3.2. ViroSeq Assay

The ViroSeq assay was evaluated for accuracy and reproducibility using a series of 3 external proficiency panels from the NIH VQA program. Fourteen samples, (5 from VQA panel 4:004g01-004g05; 4 from VQA panel 5: 005g01, 005g03, 005g04 and 005g05; and 5 from VQA panel 6: 006g01-006g05) were successfully amplified and sequenced (Appendix B-D).

The mutation lists and sequence files (fasta files) were compared to results from 35 other laboratories. The mutation lists attained in our laboratory were identical to other sites participating in the trial. The scoring and analysis of the VQA panels changed after panels

4 and 5 and the sixth VQA panel was scored according to a different set of reporting rules. The results indicated that overall homology of the sequences generated in our laboratory compared to the consensus sequences were 99% for 006g01; 99.2% for 006g02; 99.3 for 006g03; 99.2% for 006g04 and 96.8% for 006g05. Moreover, 100% homology was obtained for the mutation lists, which indicates the amino acids that confer ARV drug resistance. This means that all mutations which result in resistance to antiretroviral drugs were correctly identified. The evaluation was thus found to be successful and the panel was certified. This indicates that the ViroSeq Genotyping System is reproducible and accurate in other laboratories worldwide, including our laboratory in South Africa.

The 14 reference sequences were subtyped using the Stanford HIV resistance database (www.hivdb.stanford.edu). The subtypes were as follows: 13 HIV-1 subtype B (004g01-03, 004g05, 005g01-05 and 006g01-05) and one recombinant virus CRF02_AG (004g04). These samples from the VQA panel 4 and 5 were used in the OLA assay to determine the efficiency of the assay in HIV-1 subtype B.

Nine samples, sent to Contract Laboratory Services (CLS) for routine testing, were genotyped in our laboratory. The sequences were sent to the Stanford database for subtype analysis (Table 3.2). Furthermore, it was recorded which of the seven primers used in cycle sequencing bound and produced a sequence (Table 3.2).

Furthermore, the 6 samples previously sequenced and analysed by the NICD (DR001-0102; Table 2.1) were re-sequenced and analysed. The mutation profiles were identical to those reported by the NICD.

Table 3.2: Samples sequenced in the laboratory, indicating subtype and primers resulting in successful sequence amplification:

Patient	Subtype	A	B	C	D	F	G	H
1	C	X	X			X	X	X
2	B	X	X	X	X	X	X	X
3	C	X	X			X		
4	C	X	X	X		X	X	
5	A	X	X	X		X	X	X
6	C	X	X	X		X	X	X
7	C	X	X	X		X	X	
8	C	X	X	X		X	X	X
9	C	X	X	X		X	X	X
Total		**9/9**	**9/9**	**7/9**	**1/9**	**9/9**	**8/9**	**6/9**

3.3. Conventional PCR:

3.3.1. *PCR amplification of DNA:*

3.3.1.1. Using the primers supplied in the OLA kit:

The OLA primers were initially used to amplify the subtype B control DNA (8E5; Figure 3.2a). These primers (first round: RTA, PRA and second round: RTB, PRB) were able to successfully amplify an 1197base pair (bp) fragment from the DNA control (Figure 3.2a). However, attempts to amplify the extracted DNA from 8 samples (NHWB 2; 5-9 and 14; Table 2.1) using the same protocol, were unsuccessful (Figure 3.2b). Further attempts to optimize the PCR reaction resulted in only one (NHWB 8) of 7 samples being amplified.

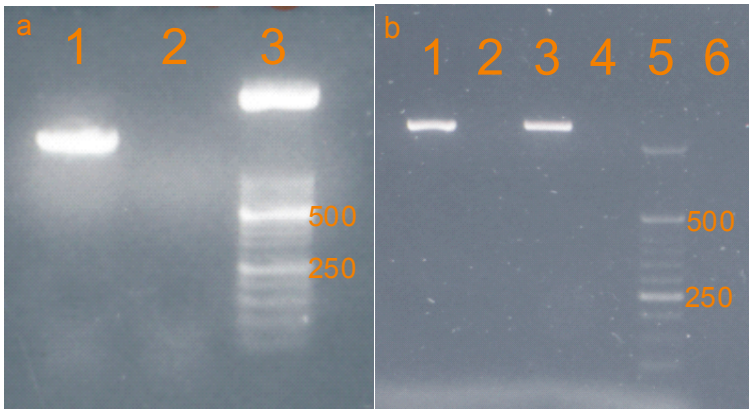


Figure 3.2: PCR amplification of extracted DNA using the OLA primers (RTA, PRA and RTB, PRB). **a)** Amplification of the subtype B8E5 DNA control. Lane 1: DNA control; Lane 2: PCR negative control; Lane 3: Molecular Weight Marker XIII (Roche, Germany). **b)** Amplification of Subtype C samples. Lane 1: DNA control; Lane 2: NHWB 14; Lane 3: NHBW 8, Lane 4: NHWB 9; Lane 5: Molecular Weight Marker XIII (Roche, Germany); Lane 6: HIV-1 negative sample. Molecular Weights (bp.) are indicated next to the ladder.

3.3.1.2.Using the primers A-(35) and NE-1(35) and ST1 and ST2

3.3.1.2.1. Nested Reaction:

First round primers A-(35) and NE-1(35) and second round primers ST1 and ST2 were used to successfully amplify a 543bp fragment from the DNA control (8E5) and the subtype C test samples (Figure:3.3).

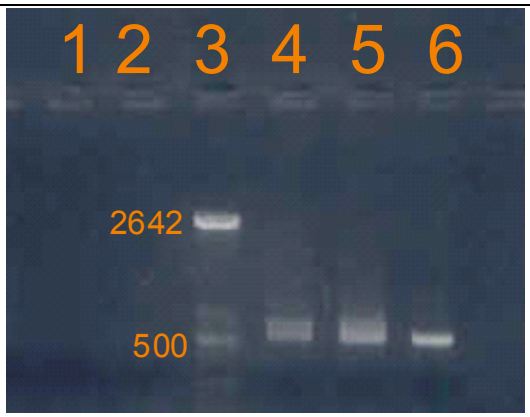


Figure 3.3: Amplification using the A-(35) & NE-1(35) primers for first round and the ST1 & ST2 for second round. Lane 1: HIV-1 negative sample; Lane 2: PCR negative control; Lane 3: Molecular Weight Marker XIII (Roche, Germany); Lane 4: DNA control; Lane 5; NHWB 20; Lane 6: NHWB 14. Molecular Weights (bp.) are indicated next to the ladder.

3.3.1.2.2. Single Round Amplification using primers ST1 and ST2

The subtype C specific primers were used in a single round PCR amplification step to successfully amplify the 543bp fragment from 14 extracted DNA samples. An example of the successful amplification of these samples is shown in Figure 3.4.

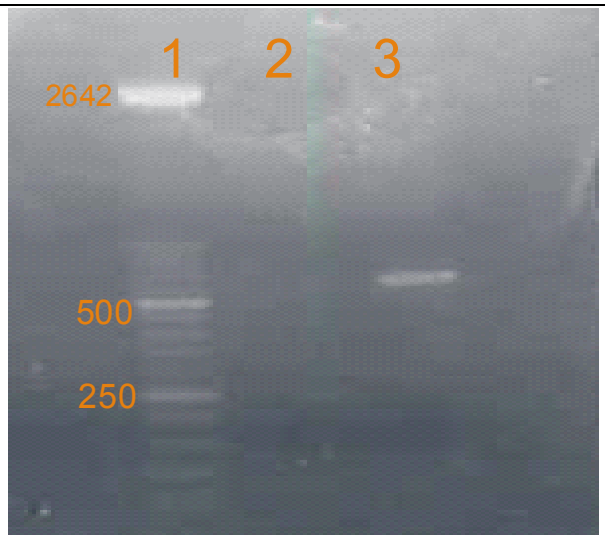


Figure 3.4: Single round PCR amplification of extracted DNA using ST1 & ST2 primers. Lane 1: Molecular Weight Marker XIII (Roche, Germany); Lane 2: PCR negative control; Lane 3: NHWB 10. Molecular Weights (bp.) are indicated next to the ladder.

3.3.2. Amplification of RNA:

The PCR reactions were successfully optimized on the extracted DNA. However, in a clinical setting the desirable starting material is viral RNA and thus a RT step needed to be optimized.

3.3.2.1.<u>One and Two-step RT-PCR using OLA primers</u>

Extracted viral RNA from 21 HIV-1 plasma samples (NHP1-14, DR95, DR94, DR47, DR16, DR50, and DR37) was used to perform the one and two-step RT-PCR using the OLA primers. The two-step RT-PCR was similar to that performed by Beck et al., (2002),

however amplification was unsuccessful. Similarly, when a one-step RT-PCR was performed no amplification was obtained. In an attempt to optimize both the RT-PCR reactions, the following steps were taken: a) the amount of viral RNA used to generate cDNA was titrated from 2ul to 35ul, b) the amplicon carried over from the first round to the second round was increased from 2ul to 10ul, c)the first round amplicon carried over was also diluted 1 in 10 and 2ul added to the second round ,d) the $MgCl_2$ was titrated from a concentration of 1.5mM to 2.5mM, to vary the salt concentration in the PCR reaction, and e) the amplification conditions were made less stringent by decreasing the annealing temperature from 55ºC to 50ºC; increasing the elongation, annealing, denaturation time and cycle number and finally the OLA primers were re-synthesized to ensure no technical error had occurred during primer synthesis.

3.3.2.2. One-step RT-PCR using primers A-(35) and NE-1(35) primers followed by a nested PCR using ST1 and ST2 primers

The extracted viral RNA from 10 samples (DR50, DR37, NHP1-2, NHP5-10), was successfully amplified using primers A-(35), NE-1(35) for first round (800bp amplicon; Figure 3.5) and ST1, ST2 for second round (543bp amplicon). This PCR reaction was found to be able to amplify HIV-1 subtype C and B samples (DR001-0102; NHP1-25) as well as the DNA control (8E5) and plasmids (p8E5, pNVP, p65R).

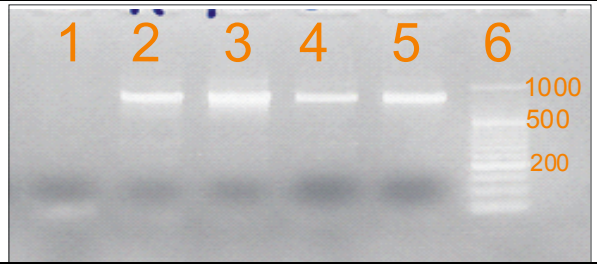


Figure 3.5: PCR amplification of viral RNA using the A-(35) and NE1-(35) primers in a one-step RT-PCR. Lane 1: PCR negative control; Lane 2: DR44; Lane 3: DR47; Lane 4: DR68; Lane 5: DR69; Lane 6: O' RangeRuler™ 50bp DNA Ladder (Fermentas, Lithuania). Molecular Weights (bp.) are indicated next to the ladder.

3.3.2.3. One-step RT-PCR using primer ST1 and ST2

The sensitivity of the one-step RT-PCR was evaluated on viral RNA extracted from 14 samples (NHP 12-25) with varying viral loads. This was achieved by using the ST1 and ST2 primers. The results indicated that amplification was limited by the plasma viral load and not the amount of extracted viral RNA used as starting material (Figure 3.6). Viral loads below 2450 copies/ml were poorly amplified.

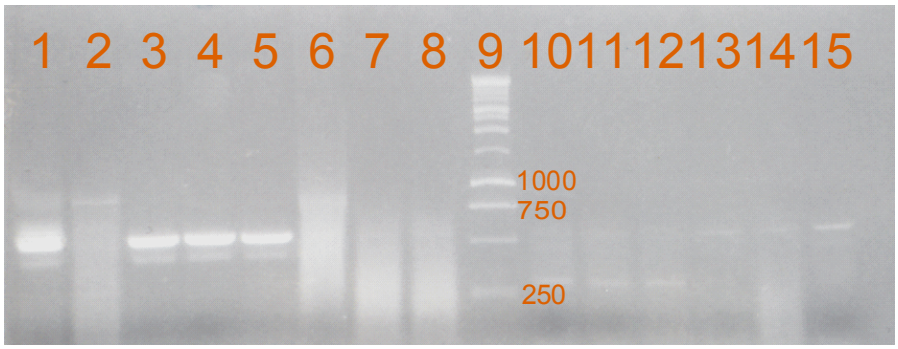


Figure 3.6: One-step RT-PCR amplification, using primers ST1 & ST2 in samples with differing viral loads. Lane 1: Pooled HIV-1 subtype C samples all with viral loads >750000 copies/ml. Lane 2: NHP 14 with 4870 copies/ml. The following lanes have different amounts of starting material: a) 10ul, b) 8ul and c) 6ul of viral RNA. Lanes 3-5: NHP17 with viral load >750000copies/ml; Lanes 6-8: NHP13 with viral load 2450 copies/ml; Lane 9: GeneRuler™ 1kb DNA ladder (Fermentas, Lithuania); Lanes 10-12: NHP16 with viral load 1610 copies/ml; Lanes 13-14: NHP15 with viral load 3400 copies/ml. Molecular Weights (bp.) are indicated next to the ladder.

3.3.3. Amplification of Controls:

The HIV-1 subtype B plasmids were successfully amplified using two different primer sets. Firstly, the primers RT3 and PRC amplified a 1060bp amplicon and the amplicon's concentration was determined using a mass ladder (Figure 3.7). Secondly, the ST1 and ST2 primers amplified a 543bp product from the DNA control (8E5), plasmids p65R, p3TC, p151M, pMDRI (Table 2.4; Figure 3.8).

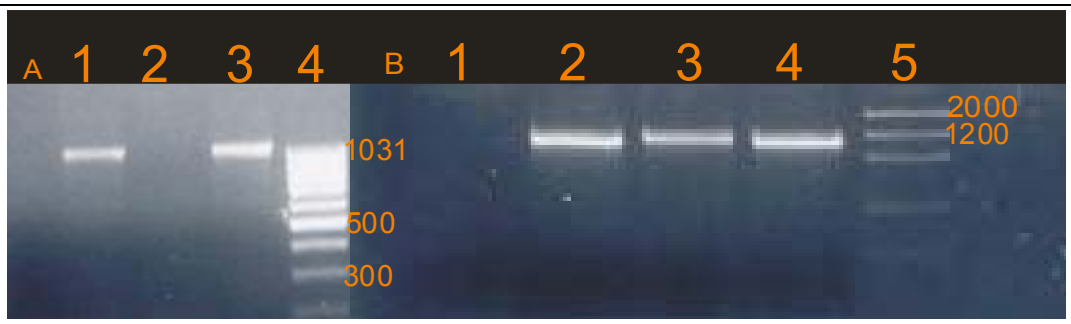


Figure 3.7: PCR amplification of plasmids using RT3 and PRC primers. **A)** Lane 1: p65R; Lane 2: PCR negative control; Lane 3: p8E5; Lane 4: MassRuler^{TM} DNA Ladder, Low Range (1031bp=50ng; 500bp=50ng; 300bp=15ng; Fermentas, Lithuania), concentration refers to 5ul of loaded sample. **B)**: Lane 1: PCR negative control; Lane 2: pNVP; Lane 3: p8E5; Lane 4: p151M; Lane 5: 3ul of the ViroSeq Mass Ladder (2000bp=50ng; 1200bp=30ng; Celera Diagnostics, USA). Molecular Weights (bp.) are indicated next to the ladder.

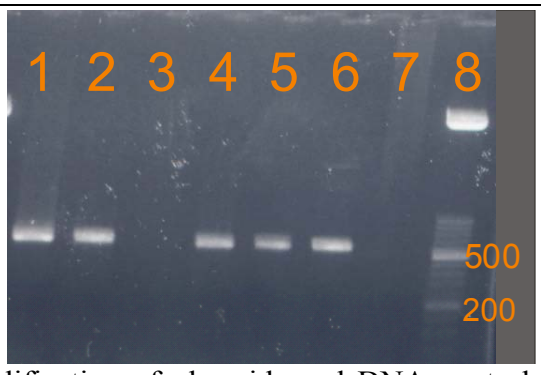


Figure 3.8: PCR amplification of plasmids and DNA control using the ST1 & ST2 primers. Lane 1:8E5 DNA control from the OLA kit; Lane 2: p65R; Lane 3: HIV-1 negative control; Lane 4: p3TC; Lane 5: p151M; Lane 6: pMDRI; Lane 7: PCR negative control; Lane 8: Molecular Weight Marker XIII (Roche, Germany). Molecular Weights (bp.) are indicated next to the ladder.

3.3.4. Comparison of PCR amplification using the OLA and ST1 and ST2 primers on HIV-1 subtype C samples

The amplification of extracted viral RNA of 12 samples (NHP 5-16) using two different primer sets (OLA and ST1 and ST2) was compared. The amplification was unsuccessful using the OLA primers; whereas the ST1 and ST2 primers were able to amplify the 543bp fragment from the HIV-1 subtype C samples (Figure 3.9).

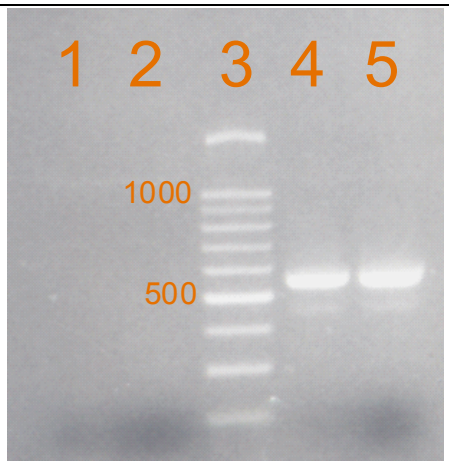


Figure 3.9: Comparison of amplification using the OLA and ST1 and ST2 primers. This amplification was performed on three pooled HIV-1 subtype C samples with viral loads >750000 copies/ml. Lane 1-2: OLA primers; Lane 3: O' RangeRuler^{TM} 100bp DNA Ladder (Fermentas, Lithuania).; Lane 4-5: ST1 and ST2 primers. Molecular Weights (bp.) are indicated next to the ladder.

3.3.5. Amplification of ViroSeq amplicons:

Successful second round amplification of the 1.8bp amplicons (1:10 diluted ViroSeq product) was achieved using the ST1 and ST2 primers (543bp; Figure 3.10) or the A-(35) and NE1-(35) primers (800bp, results not shown).

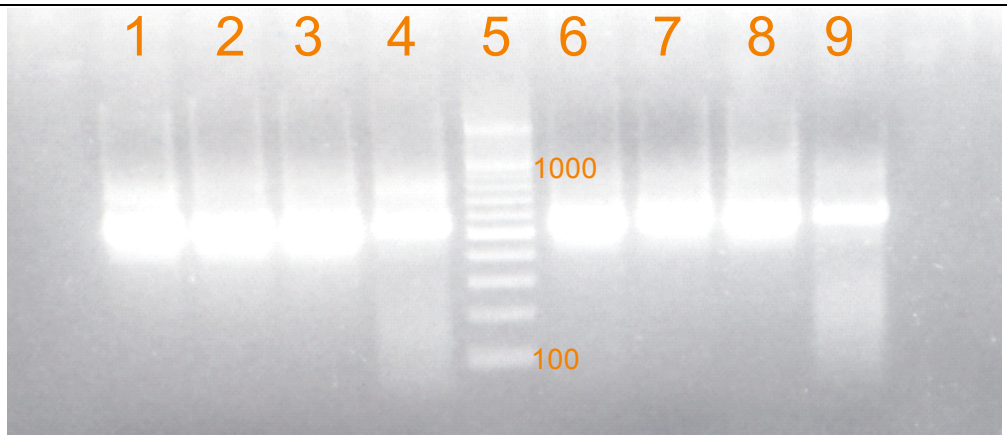


Figure 3.10: HIV-1 PCR products amplified using ST1 and ST2 primers. Lanes 1-4 undiluted PCR amplicon; Lanes 6-9 a 1:10 diluted amplicon. Lane 1: DR 94; Lane 2: DR 95; Lane 3: NHWB14 (see section 2.4.1.2); Lane 4: DR49; Lane 5: O' RangeRuler^{TM} 50bp DNA Ladder (Fermentas, Lithuania); Lane 6: DR 94; Lane 7: DR95; Lane 8: DR22; Lane 9: DR49. Molecular Weights (bp.) are indicated next to the ladder.

3.3.6. Gel Elution

The amplified 543bp product of the amplified ViroSeq product, plasmids and DNA control were successfully gel eluted and used as the starting material for the MGB assay (Figure 3.11).

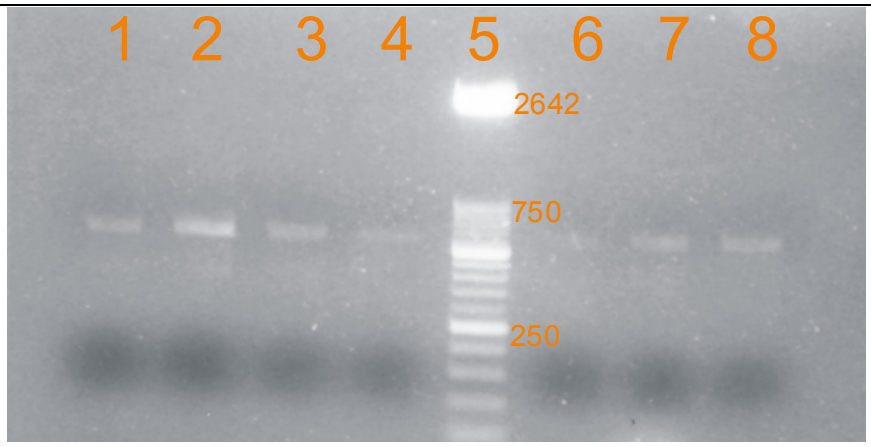


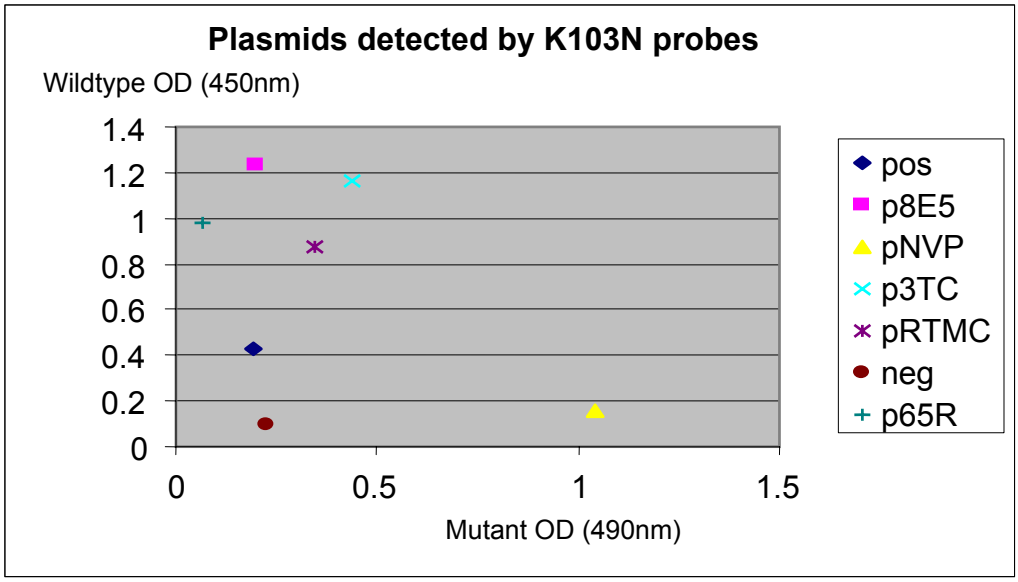
Figure 3.11: Eluted PCR products amplified from plasmids and DNA control. Lane 1: p3TC; Lane 2: DNA control (8E5); Lane 3: pMDRI; Lane4: p65R; Lane 5: Molecular Weight Marker XIII (Roche, Germany); Lane 6: pNVP; Lane 7: p151M; Lane 8: p8E5. Molecular Weights (bp.) are indicated next to the ladder.

3.4. Alternative Methodology evaluation

3.4.1. *Oligonucleotide Ligation Assay (OLA)*

The amount of PCR product (see section 2.4.4.2) used for detection was increased from the recommended 2ul to 5ul. This allowed for better detection of the controls; namely DNA control (8E5), the wild-type plasmid (p8E5) and the mutant plasmids (p65R and pNVP; Table 2.4). All assays where repeated in triplicate, and the results obtained were similar, which indicated the assay was performed correctly. The controls were included and correctly detected in all the OLA detection assays performed (Figure 3.12; Table 3.3). Furthermore, the p3TC, pRTMC, pMDR1 and p151M plasmids (all K65, K103 and Y181 genotyped) were successfully analysed. The wild-type and mutant cut-offs were determined by calculating the standard deviation for either the wild-type or mutant from the ODs of blanks in each assay and subtracting these from 2.5 (Beck, et al., 2002).

a



b

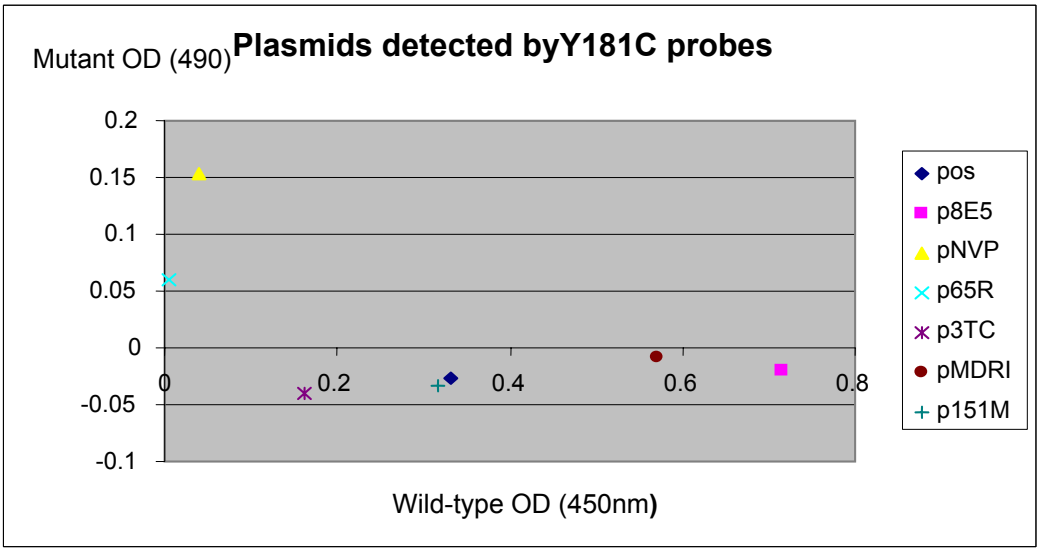


Figure 3.12: Scatter plots of OD readings indicating that all the plasmids for the **a)** K103N and **b)** Y181C mutation were correctly detected. The wild-type genotype is depicted by a high wild-type OD and low mutant OD readings, whereas mutant is shown as a low wild-type OD and a high mutant OD Reading.

Table 3.3: Summary of the codons analysed with OLA.

Codon	K103N (NNRTI kit)			Y181C (NNRTI kit)			K65R (NRTI kit)		
	Plasmid	VQA	Sub-type C	Plasmid	VQA	Sub-type C	Plasmid	VQA	Sub-type C
Total Samples	6	9	20	6	9	17	6	9	19
No. of Wild-type	5	3	9	5	9	14	5	9	15
No. of Mutant	1	6	11	1	0	3	1	0	4
Total no. correctly identified	6	2	5	6	4	4	6	3	0
Percentage correctly identified	100%	22%	25%	100%	44%	24%	100%	33%	0%

3.4.1.1. VQA samples

Nine VQA samples (004g01-05 and 005g01, 005g03-05) were used for detection of codons: K103N, Y181C and K65R. The codons were detected as follows: 2/9 for Y103N; 4/9 for Y181C and 3/9 for K65R (Table 3.3).

For codon 103, of the 9 samples, 3 were wild-type and 6 were mutant as determined by genotyping. However, only 2 of these samples (005g03 and 005g05) were correctly identified by OLA (Table 3.3). Both of these samples had wild-type sequences that showed 100% homology to the probe (Figure 3.13a). The third wild-type sequence (004g04) had 5 mismatches compared to the probe. Of the 6 mutant sequences 4 had the unusual K103S mutation (004g02, 004g03, 004g05, and 005g01) as well as 3 other mismatches that probably contributed to either no binding or low binding to both wild-type and mutant probes. Both K103N mutant sequences (004g01 and 005g02) had 4 mismatches compared to the probe.

Similarly, for codon 181, all 9 samples were wild-type as determined by genotyping. However, only 4 of these samples (004g03, 005g02, 005g03, and 005g05) were correctly identified by OLA (Table 3.3), 3 (004g03, 005g03, and 005g05) of which had wild-type sequences that had 3-4 mismatches at a significant distance from the ligation site. The remaining wild-type sequence correctly detected (005g02) had 100% homology to the probe (Figure 3.13b). Of the non-detected wild-type sequences, sample 004g01 had three mismatches compared to the probe, probably resulting in no binding, or insufficient binding of this probe resulting in poor detection. Samples 004g02, 004g04 and 004g05 had 5-8 mismatches compared to the probe, with one of them residing at the ligation site resulting in no probe binding. Moreover, sample 004g04 also had an insertion of 2 adenine bases. Finally, the mutant sample 005g01 had 1 mismatch close to the ligation site and was not detected.

Similar sensitivities were reported for the mutations detected in the NRTI kit using the VQA samples. Of the 9 samples evaluated for codon 65, all were wild-type as determined by genotyping. However, only 3 of these samples (004g03, 004g04 and 005g01) were correctly identified by OLA (Table 3.3). Sample 004g04 had 2 mismatches on the common probe 6 bases from the ligation site, samples 004g03 and 005g01 had 4 and 5 mismatches respectively, but these were spaced evenly throughout the probe. Samples 004g02 and 004g05 were both not detected and had 6 mismatches compared to the probes (Figure 3.13c). The remaining 4 samples had mismatches close to the ligation site as well as other mismatches which would have resulted in no or poor probe binding.

3.4.1.2. OLA detection results for HIV-1 subtype C samples

Twenty two Subtype C samples were selected from a cohort of 102 patients referred for routine genotypic resistance testing (in-house numbering: DR001-102). The subtype C samples were tested for the same codons as the subtype B samples described above. For codon 103, of the 20 subtype C samples, 9 were wild-type and 11 were mutant as determined by genotyping. However, only 5 of these samples were correctly identified by OLA (Table 3.3), of which 4 sequences were obtained (DR010, DR017, DR037 and DR094). Samples DR010, DR017 and DR037 had wild-type sequences that showed 2-3 mismatches to the probe (Figure 3.14a). The fourth correctly identified sequence (DR094) had a mutant sequence that revealed 3 mismatches compared to the probe. The 5 undetected wild-type sequences (DR016, DR019 and DR044) had 7, 6 and 4 mismatches compared to the probe, whereas DR084 had a mismatch one base from the ligation site and DR029 had a mismatch at the ligation site, both had 4 mismatches compared to the probe (Figure 3.14a). Of the 4 sequences obtained for undetected mutants, (DR028, DR047, DR050, DR095) it was found that mismatches 4-5 compared to the probe and these mismatches occurred close to the ligation site.

For codon 181, of the 17 subtype C samples, 14 were wild-type and 3 were mutant as determined by genotyping. However, only 4 of these samples were correctly identified by OLA (Table 3.3) Two of these samples (DR029 and DR068) had wild-type sequences that showed 4 and 6 mismatches to the probe, respectively (Figure 3.14b). One sample (DR012) was incorrectly detected as a false positive and upon sequence analysis was found to have mismatches on both sides of the ligation site, resulting in sub-optimal ligation. 11/17 samples were not detected and of the 10 sequences available on these samples

(DR006, DR084, DR048, DR041, DR095, DR094, DR044, DR037 and DR028), analysis revealed that mismatches ranged from 6-29 compared to the probe. Moreover a mutant sample (DR021) had 4 mismatches compared to the probe close to the ligation site (Figure 3.14b).

Of 19 subtype C samples, 15 were wild-type and 4 mutants for codon 65. 0/19 samples were detected correctly (Table 3.3). Sequence analysis revealed that no probe binding was a result of a consistent base change two bases away from the ligation site on the wild-type/mutant probes of samples DR010, DR016, DR019, DR028, DR029, DR037, DR050, DR084, DR006, DR092, DR095, DR098, DR044, DR068, DR069 and DR047. Sample 17 had 10 mismatches compared to the probe. Moreover, all samples with a mismatch close to the ligation site also had mismatches ranging in number from 4-31 (Figure 3.14c).

a) K103N:		Mismatches: Wild-type	Common
wild-type:	ACA TCC CGC AGG GTT AAA AAA GAA **A*** AAA TCA GTA ACA GTA CTG GAT GTG GGT		
Samples correctly identified by OLA:			
005g03	ACA TCC CGC AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA CTR GAT GTG GGT	0	0
005g05	ACA TCC CGC AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA CTA GAT GTG GGT	0	0
Samples not correctly identified by OLA:			
004g01	ACA TCC TGC AGG GAT AAA AAR GAA C *AAA TCA GTA TCA GTA ATA GAT GTG GGT	2	2
004g02	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
004g03	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
004g04	TCA TCC CGC GGG ATT AAA AGA GAA A *AAA TCG GTA ACA GTA CTA GAT GTG GGG	3	2
004g05	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
005g01	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
005g02	ACA TCC TGC AGG GAT AAA AAR GAA C *AAA TCA GTA TCA GTA ATA GAT GTG GGT	2	2
b) Y181C:			
wild-type:	ACA AAA TCC AGA CAT AGT TAT CTA **A** *TCA ATA CAT GGA TGA TTT GTA TGT A		
Samples correctly identified by OLA:			
004g03	ACA GAA TCC AGA AAT AGT TAT CTA *TCA ATA YGT GGA TGA TTT GTA TGT A	2	2
005g02	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CAT GGA TGA TTT GTA TGT A	0	0
005g03	AAA GAA TCC AGA CAT RGT TAT CTA *TCA ATA CGT GGA TGA TTT GTA TGT A	3	1
005g05	AAA GAA TCC AGA CAT RGT TAT CTA *TCA ATA CGT GGA TGA TTT GTA TGT A	3	1
Samples not correctly identified by OLA:			
004g01	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CHT RGA TGA TTT GTA TAT A	0	3
004g02	ACA GAA TCC AGA AAT ART TAT CTA *YCA ATA TGT GGA TGA TTT GTA TGT A	3	3
004g04	ACA AAA AATCC AGA GAT GGT GAT CTA* CCA ATA TAT GGA TGA TTT ATA TGT A	5	3
004g05	ACA GAA TCC AGA AAT ART TAT CTA* YCA ATA TGT GGA TGA TTT GTA TGT A	3	3
005g01	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CCT GGA TGA TTT GTA TGT A	0	1
c) K65R:			
wild-type:	CTC CAG TAT TTG CCA TAA AGA **A*** RAA AGA CRG TAC TAA ATG GAG AA		
Samples correctly identified by OLA:			
005g02	CTC CAG TAT TTG CTA TAA AAA A *GAA AAA CAG TAC TAA ATG GAG AA	2	1
005g03	CTC CAG TAT TTG CCA TAA AGA A *AAA GGA CAG TAC TAA ATG GAG AA	0	1
005g05	CTC CAG TAT TTG CCA TAA AGA A *AAA GGA CAG TAC TAA ATG GAG AA	0	1
Samples not correctly identified by OLA:			
004g01	CTC CAA TAT TTG CNA TAA AGA A *GAA AAA CAG TAC TAA ATG GAG AA	1	1
004g02	CTC CAA TAT TTG CTA TAA AGA A *GAA AAA CAG TGA TCA ATG GAG AA	2	4
004g03	CTC CAA TAT TTG CNA TAA AGA A *GAA AAA CAG TGA TAA ATG GAG AA	1	3
004g04	CTC CAG TAT TTG CCA TAA AGA A *AAA AGA TAG TAC CAA ATG GAG AA	0	2
004g05	CTC CAA TAT TTG CTA TAA AGA A *GAA AAA CAG TGA TCA ATG GAG AA	2	4
005g01	CTC CAA TAT TTG CTA TAA AAA A *GAA AAA CAG TAC TAA ATG GAG AA	3	2

Figure 3.13: Nucleotide sequences of the VQA samples used in OLA detection assay: **a)** K103N, **b)** Y181C, **c)** K65R. The first line for each mutation shows the wild-type & common probe sequences (underlined), the sequences are separated into those that were correctly identified and those that could not be correctly identified, the bold base indicates the site of mutation, the asterisk the ligation site, the shaded bases reflect differences from the probes. The numbers of mismatches relative to the wild-type and common probes are indicated on the right, and the mismatches that occur near the ligation site are highlighted.

		Mismatches: Wild-type	Common
a) K103N:			
wild-type:	ACA TCC CGC AGG GTT AAA AAA GAA **A** *AAA TCA GTA ACA GTA CTG GAT GTG GGT		
Samples correctly identified by OLA:			
DR010	ACA CCC AGG AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA CTG GAT GTG GGG	3	0
DR017	ACA TCC CGC AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA ATA GAT GTG GGG	0	2
DR037	ACA CCC AGC AGG GTT AAA AAA GAA A *AAA TCA GTG ACA GTA CTG GAT GTG GGG	2	1
DR094	ACA CCC AGC AGG GTT AAA AAA GAA C *AAA TCA GTG ACA GTA CTG GAT GTG GGG	2	1
Samples not correctly identified by OLA:			
DR016	ACA CCC AGG AGG GTT AAA AAA GAA A *AAA TCA GTG ACA GTA CTA GAT ATA GGG	3	4
DR019	ACA CCC AGC AGG GTT AGA GAA GAA A *AAA TCA ATG ACA GTA CTG GAT GTG GGG	4	2
DR028	ACA TCC AGG AGG ATT AAA AAA GAA C *AAA TCA GTG ACA GTA CTA GAT GTG GGG	3	2
DR029	ACA CCC AGC AGG GTT AAA AAA GAA T *AAA TCA GTG ACA GTA CTG GAT GTG GGG	3	1
DR047	ACA CCC AGC AGG GTT AAA GAA GAA C *AAA TCA ATG ACA GTA CTG GAT GTG GGG	3	2
DR050	ACA CCC AGC AGG GTT AAA AAA GAA C *AAA TCA ATG ACA GTA CTG GAT GTG GGG	2	2
DR084	ACA CCC AGC AGG ATT AAA AAA GAG A *AAA TCA GTA ACA GTA CTG GAT GTG GGG	4	0
DR095	ACA TCC AGC AGG GTT AAA AAA GAA C *AAA TCA ATG ACA GTA CTG GAC GTG GGG	1	2
DR044	ACA CCC AGC AGG GTT AAA AAA GAA A *AAA TCA GTG ACA GTA CTA GAT GTG GGG	2	2
b) Y181C:			
wild-type:	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CAT GGA TGA TTT GTA TGT A		
Samples correctly identified by OLA:			
DR029	ACA AAA TCC AGA AAT AGT CAT CTA *TCA ATA TAT GGA TGA CTT GTA TGT A	2	2
DR068	AAA AAA TCC AGA CMT AGT TAT CTA *TCA ATA TGT GGA TGA CTT RTA TGT A	2	4
Samples not correctly identified by OLA:			
DR006	ACG AAA CCA AGA AAT AGA GAT CTG *TCA ATA CAT GGA TGA CTT GTA TGT A	6	1
DR012	ACA AAA TCC AGA CAW AGT TAT CTG *TCA ATA TAT GGA TGA TTT GTA TGT A	1	1
DR021	ACA AAA TCC AGA AGT AGT TAT CTG *TCA ATA CGT GGA TGA TTT RTA TGT A	2	2
DR084	AAA AAA CCC AGA CAT AGA TAT CTA *TCA ATA TAT GGA TGA CTT GCT TGT A	3	4
DR048	GAA AAC AAA ATC CAG ACA TAG TCA *TTT RTC AAT ACA TGG ATG ATT TGT A	14	12
DR041	GGG CAC AAA ATC CAG AMA TAG TCA *TCT GTC AAT ACG TGG ATG ACT TGT A	16	13
DR095	AAA AAA TCC AGA AAT AGT CAT TTA *TCA ATA TAT GGA TGA CTT ATA TGT A	5	3
DR094	ACA AAA CCC AGA AAT AGT CAT CTA *TCA ATA TGT GGA TGA CTT GTA TGT A	3	3
DR044	AAA AAA TCC AGA ACT AGT CAT CTA *TCA ATA TAT GGA TGA TCT GTA TGT A	4	2
DR037	GAG CAA AAA ATC CAG ACA TAG TCA *TCT ATC AAT ATG TGG ATG ACT TGT A	15	12
DR028	ACA AAA TCC AGA GAT AGT CAT CTA *TCA ATA TGT GGA TGA CTT GTA TGT A	2	3
c) K65R			
wild-type:	CTC CAG TAT TTG CCA TAA AGA **A***RAA AGA CRG TAC TAA ATG GAG AA		
Samples not correctly identified by OLA:			
DR010	CAA TAT TTG CCA TAA AAA AGA A*ACA GTG ATA AGT GGA GAA AAT TA	12	19
DR016	CTC CAG TAT TTG CCA TAA AAA A*GAA GAA CAG TGA TAA GTG GAG AA	1	6
DR017	CTC CAA TAT TTG CTA TCA AGA A *AAA GAA CAG CGA TAG ATG GAG AA	3	7
DR019	CTC CAG TAT TTG CCA TAA AAA A*GAA AAA CTC TGA GAG TTC TAA GTG GAG AA	1	22
DR028	CTC CAG TAT TTG CTA TAA AAA A*GAA AGA CAG TAC TAA GTG GAG AA	2	2
DR029	CTC CAA TAT TCG CCA TAA AAA A*GAA GAA CAG CAC TAG GTG GAG AA	2	6
DR037	CCC CAR TAT TTG CCA TAA AAA A*GAA GAA CAG TAC TAG GTG GAG AA	3	5
DR050	CTC CAG TAT TTG CCA TAA AAA A*GAA GGA CAG TAC TAA GTG GAG AA	1	3
DR084	CTC CAA TAT TTG CCA TAA AAA A*GAA GGA CAG TAC TAA GTG GAG AA	2	3
DR006	CTC CAA TAT TTG CCA TAA AAA A*GAA AGA TGG MAA TAA GTG GAG AA	2	5
DR092	CTC CAA TAT TTG CCA TAA AAA A*GAA GAA CAG TGA TAG GTG GAG AA	2	7
DR095	CCC CGG TGT TTG CTA TAA AAA A*GAA GGA CAG TAC TAA GTG GAG AA	5	3
DR098	CTC CAA TAT TTG CCA TAA AAA A*GAA GAA CAG TAC TAR GTG GAG GA	2	6
DR044	CTC CAA TAT TTG CCA TAA AAA R*GAA GGA CAG TAC TAA GTG GAG AA	2	3
DR068	CTC CAG TAT TTG TCA TAA AAA R*GAA GGA CGG TAY TAA GTG GAG AA	2	4
DR069	CTC CAG TAT TTG CCA TAA AAA G*GAA GAA CAG TAC TAA GTG GAG AA	1	4
DR047	CTC CAG TAT TTG CTA TAA AAA R*GAA GRA CAG TAC TAA GTG GAG AA	2	4

Figure 3.14: Nucleotide sequences of the subtype C samples used in OLA detection assay: **a)** K103N, **b)** Y181C, **c)** K65R. The first line for each mutation shows the wild-type & common probe sequences (underlined), the sequences are separated into those that were correctly identified and those that could not be correctly identified, the bold base indicates the site of mutation, the asterisk the ligation site, the shaded bases reflect differences from the probes. The numbers of mismatches relative to the wild-type and common probes are indicated on the right, and the mismatches that occur near the ligation site are highlighted.

3.4.2. Restriction Fragment Length Polymorphism Analysis:

The restriction enzyme *MaeIII* recognizes the sequence /GTNAC which can be found in the RT gene of HIV-1. The degenerate base in *MaeIII'*s recognition sequence allows for restriction of both HIV-1 subtype B and C (subtype B, GTA and subtype C, GTG). However, when the mutation is present (subtype B, ATG and subtype C, GCA), the restriction site is abolished. Of the 22 samples analyzed using the restriction enzyme *MaeIII*, 1 sample contained V106A and 6 samples contained the V106M mutation, 18/18 samples where correctly identified using the restriction enzyme (Table 3.4). Figure 3.15 indicates PCR amplicon (543bp) before and after restriction (fragments of 408 and 135bp generated).

Table 3.4: Sample Results following restriction with enzyme *MaeIII*

Sample	Genotype	Fragments After Restriction	Predicted Fragments After Restriction	Correctly Detected
8E5	V106	2	2	Yes
NEG	0	0	0	Yes
HR22	V106M	1	1	Yes
HR25	V106	2	2	Yes
p151M	V106	2	2	Yes
p3TC	V106	2	2	Yes
pMDRI	V106A	1	1	Yes
p65R	V106	2	2	Yes
DR095	V106M	1	1	Yes
DR044	V106	2	2	Yes
DR098	V106M	1	1	Yes
DR069	V106M	1	1	Yes
DR098	V106M	1	1	Yes
DR048	V106	2	2	Yes
DR041	V106	2	2	Yes
DR037	V106	2	2	Yes
DR092	V106M	1	1	Yes
DR017	V106	2	2	Yes

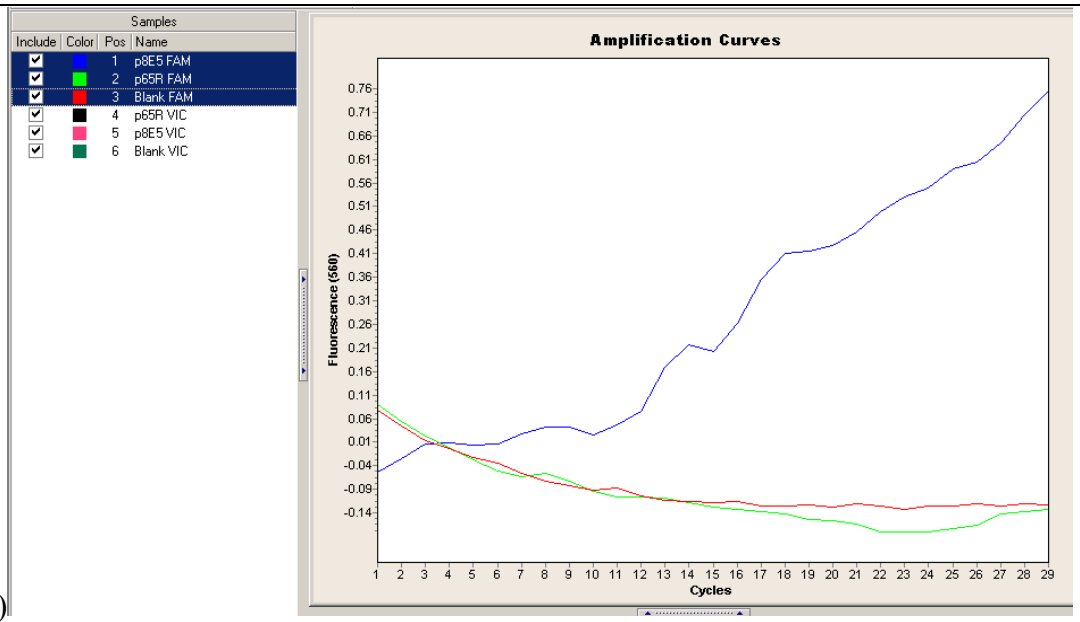
Figure 3.15: Amplified PCR product before and after restriction with *MaeIII*. The first lane for each sample is digested PCR product, and the second lane is the unrestricted starting PCR product. Lanes 1-2 pMDRI with V106A mutation and partial restriction; Lanes 3-4 DR044 with V106; Lanes 5-6 DR017 with V106; Lanes 7-8 DR048 with V106 and partial restriction; Lanes 8-9 DR041 with V106; Lane 10: O' RangeRuler™ 50bp DNA Ladder (Fermentas, Lithuania); Lanes 11-12: DR037 V106; Lanes 13-14: DR092 V106M; Lanes 15-16: DR050 with V106. Molecular Weights (bp.) are indicated next to the ladder.

3.4.3. Minor Groove Binding

Amplification using the MGB primers and probes was performed on the LightCycler II (Roche). Initially the amplification and probe binding reaction was optimized on known concentrations of subtype B plasmids (p8E5-100%wildtype or p65R-100% mutant; Figure 3.7) in separate reactions. The amplification cycles were decreased from 45 to 30 (Figure 3.16) because the negative control appeared to be emitting a signal after 40 cycles thought to be as a result of amplification stress which resulted in probe degradation. The wild-type and mutant probe were correctly detected (Figure 3.16). The known concentration of plasmid PCR product was serially diluted from 50ng to 3,125ng to determine the effect of amplicon concentration added on final fluorescence. The results indicated that a variation in starting material did not alter end point fluorescence. Probes were able to correctly classify 5% populations of either wild-type or mutant (Figure 3.17). This was determined by mixing both wild-type and mutant plasmids together in varying concentrations ranging from 100% wild-type and 0% mutant to 5% wild-type and 95% mutant.

In general, the HIV-1 subtype B samples were found to fluoresce at a lower level than that of subtype C samples (see table 2.2 for additional information), and thus HIV-1 subtype B plasmids were not used as controls. Figure 3.18 illustrates that HIV-1 subtype C samples (DR069-red, DR029-pink and DR044-green) fluoresced between OD readings of 1.3 and 1.53 (with the exception of DR047-black), whereas the HIV-1 subtype B plasmid (p8E5-bright green) fluoresced at an OD of 0.253. The optimal amount of mutant VIC probe was determined by titrating it from 0.4-0.8pmol on subtype C samples. The probe concentration appeared to make no difference to the fluorescence signal recorded at the end point; however the sample with 0.6pmol of VIC probe (pink) appeared to generate the smoothest curve (Figure 3.19). It was determined that the best concentration for the VIC labelled probe was 0.6pmol in a final volume of 20ul.

a)



b)

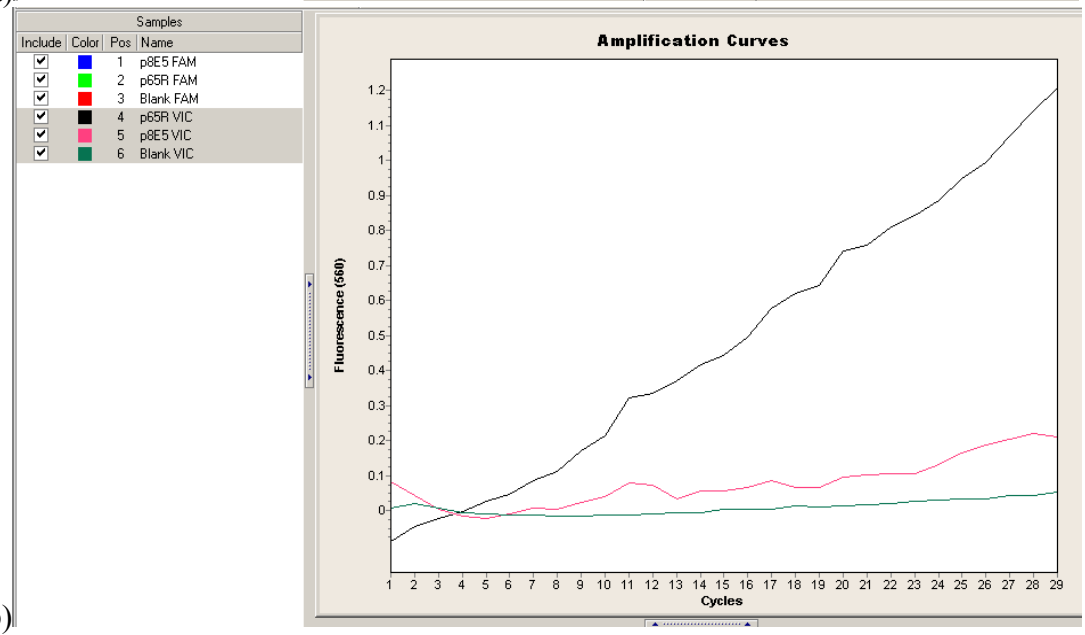
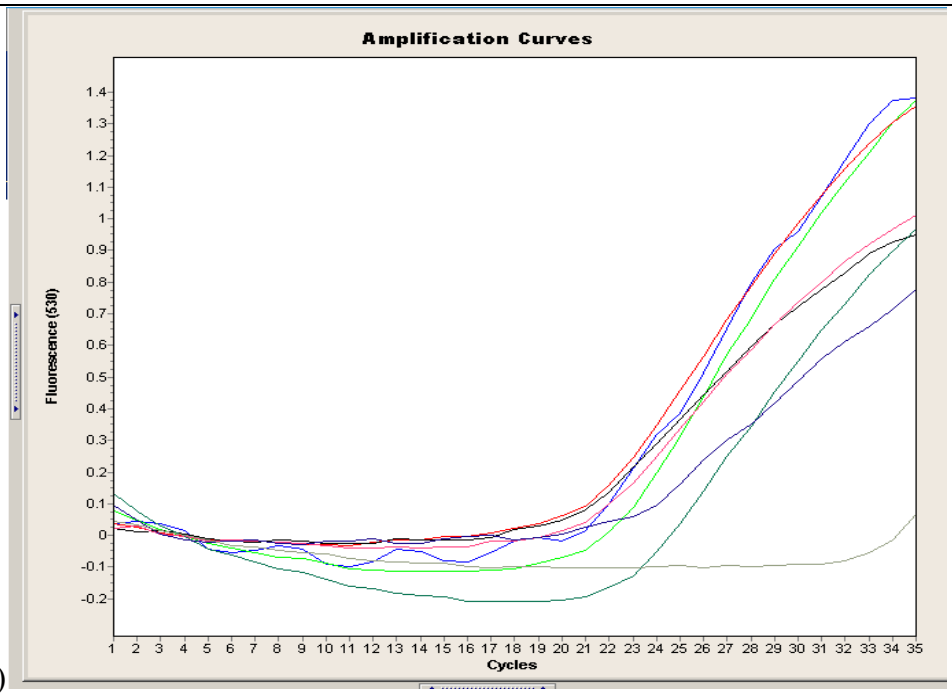


Figure 3.16: PCR amplification of HIV-1 subtype B plasmids using the K65R MGB probe. **a)** Detection using the wild-type FAM labelled probe, **b)** detection using the mutant VIC labelled probe demonstrating accurate detection of both the wild-type p8E5 and mutant p65R plasmid.

a)



b)

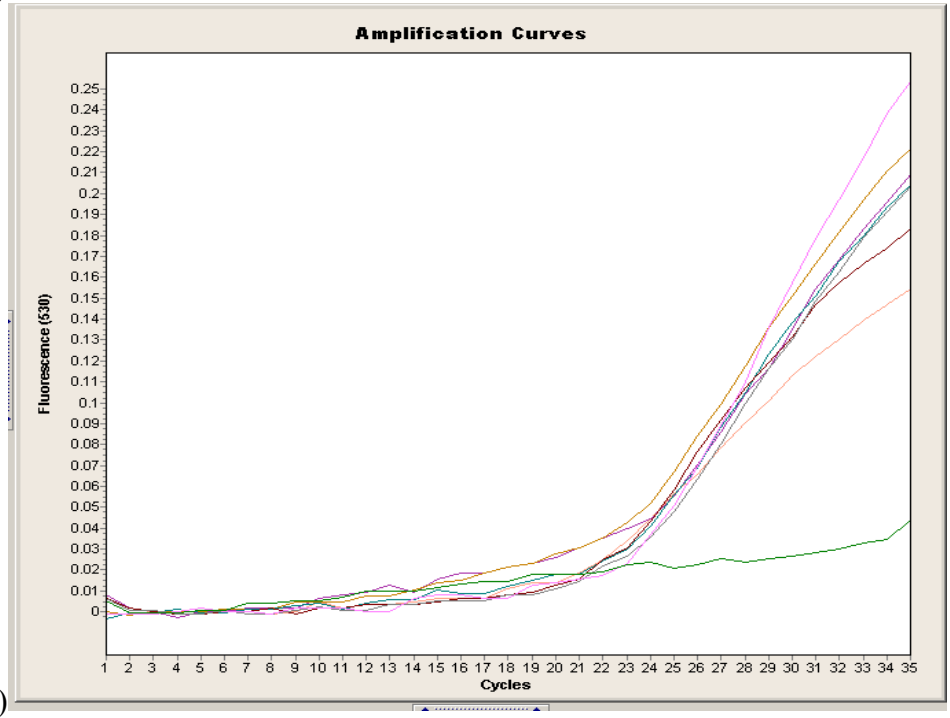


Figure 3.17: Sensitivity of the MGB probes: **a)** the detection of the wild-type probe. The blue, green and red lines represent 100%; 95% and 75% of a wild-type population. The pink and black lines represent 50% and 25% wild-type population, respectively. The dark blue line indicates 5% wild-type population, the grey line represents the blank. **b)** The detection of the wild-type probe. The pink, grey, maroon lines represent 100%; 95% and 75% of a wild-type population. The turquoise and orange lines represent 50% and 25% wild-type population, respectively. The peach blue line indicates 5% wild-type population, the green line represents the blank.

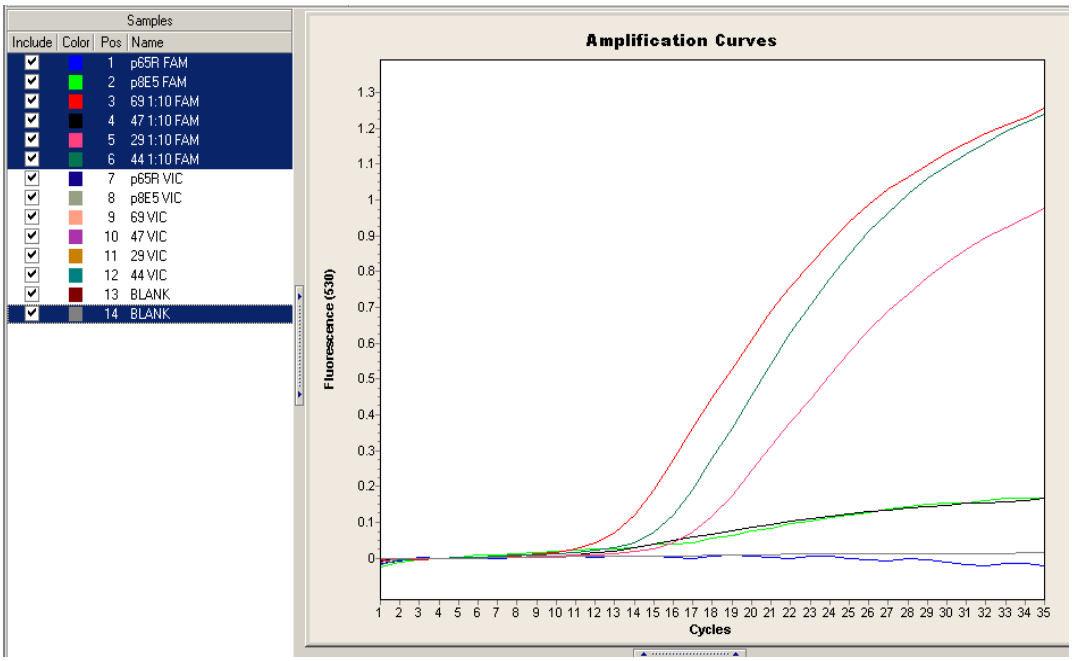


Figure 3.18: Comparison of HIV-1 subtype B and C samples (red, black, pink and green). The HIV-1 subtype C samples are detected at a higher fluorescence than the HIV-1 subtype B plasmids (bright green p8E5, royal blue p65R).The grey line indicates the blank.

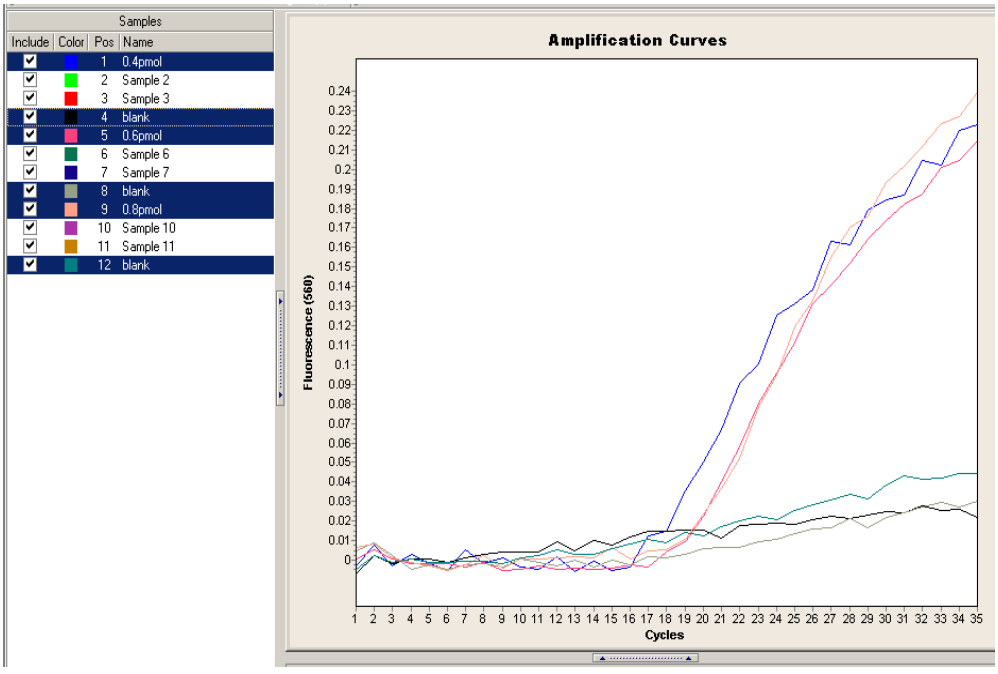


Figure 3.19: Titration of the VIC labelled MGB probe at concentrations 0.4pmol (royal blue); 0.6pmol (pink) and 0.8pmol (peach). The blanks are represented by the black; grey and turquoise lines, respectively.

A multiplex reaction was the desired form of amplification, as it would decrease the cost of the assay and the turnaround time. Due to the particular fluorescence properties of the MGB probes, a novel colour compensation was performed in order to facilitate a multiplex PCR reaction. This colour compensation was achieved using a previously genotyped sample (DR044) heterologous for the K65R mutation. The performance of this approach was confirmed on all 15 patient samples, in both single and multiplex reactions. (Figures 3.20; 3.21; 3.22; 3.23; Table 3.5).

Two annealing temperature (60 and 62ºC) were compared to determine the optimal annealing temperature. In the multiplex reaction ,an annealing temperature of 62ºC resulted in the best discrimination of the 4 mutant samples DR044, 047, 059, 069 (Table 3.5; Figures 3.20; 3.21; 3.22; 3.23). The MGB multiplex reaction with an annealing temperature of 62ºC, had 3 wild-type samples that were not detected (DR047, 048 and 068), and 4 of the 5 mutant samples correctly detected (DR044, 047, 059, 069; Figure 3.21). From these single and multiplex reactions the end point fluorescence was read on a real time fluorimeter (LightCycler 2). These results indicated that a multiplex reaction could not be used because the FAM dye seeps over to the VIC reading channel resulting in inaccurate results (Table 3.5). Two separate reactions would therefore have to be used if fluorescence was to be read on a fluorimeter that does not have the ability to perform colour compensation.

Table 3.5: Comparison of Single and Multiplex Reactions at anneal ng temperature 62°C. a) H-high, b) M-middle, c) L-low fluorescence and d) X- samples not detected. Mutant samples are highlighted in yellow; heterologous samples are highlighted in green and homologous samples are highlighted in blue.

Sample Name	Simplex		Multiplex		Fluorescence Simplex		Fluorescence Multiplex	
	FAM	VIC	FAM	VIC	FAM	VIC	FAM	VIC
DR102	M	X	M	X	1.98	1.13	2.03	2.43
DR033	H	X	H	X	2.40	1.09	2.28	2.59
DR068	X	X	X	X	1.02	1.19	1.25	1.88
DR047	X	X	X	Middle	0.91	1.01	1.24	1.90
DR059	L	H	L	H	1.61	1.35	1.72	2.33
DR048	L	X	Very L	X	1.25	1.19	1.33	1.93
DR029	H	X	H	X	2.38	1.20	2.26	2.57
DR098	H	X	H	X	2.47	1.18	2.32	2.63
DR056	H	X	H	X	2.46	1.14	2.26	2.55
DR069	L	H	L	H	1.20	1.30	1.59	2.19
DR064	H	Middle	H	X	2.30	1.25	2.26	2.57
DR044	L	H	Middle	H	1.88	1.37	1.84	2.36
DR021	Late	X	Late	X	1.70	1.17	1.56	2.02
DR051	H	X	H	X	2.22	1.20	2.51	2.45
DR006	H	X	H	X	2.17	0.77	2.10	2.42
Blank	X	X	X	X	0.89	0.95	1.12	1.69

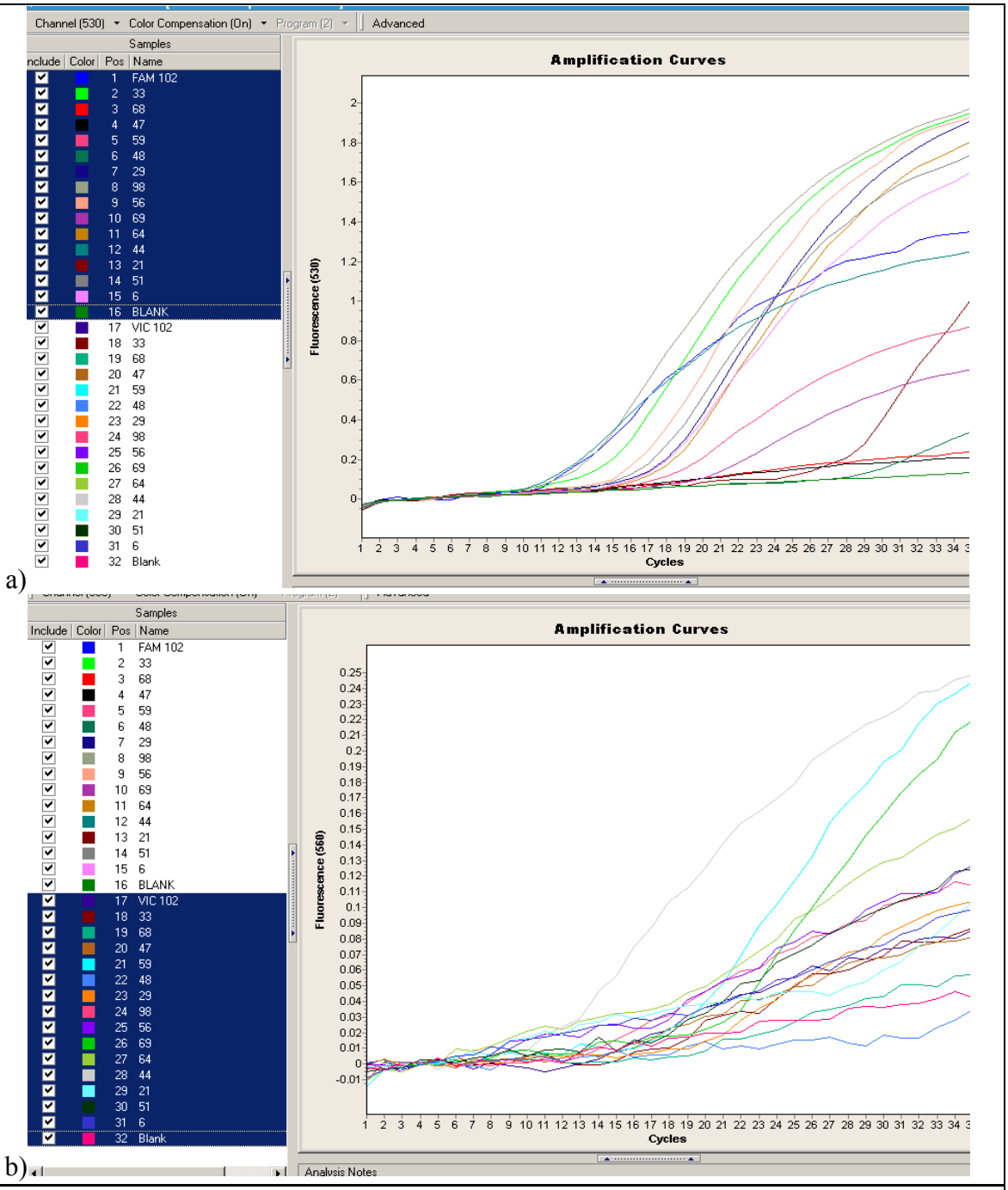


Figure 3.20: MGB single reactions for 15 HIV-1 subtype C samples at an annealing temperature of 62ºC. **a)** Wild-type samples successfully detected with the FAM probe, **b)** 4 of the 15 mutant samples DR044 (grey); DR059 (bright blue); DR069 (bright green) were detected with the VIC probe and DR064 (green) were detected with the VIC probe.

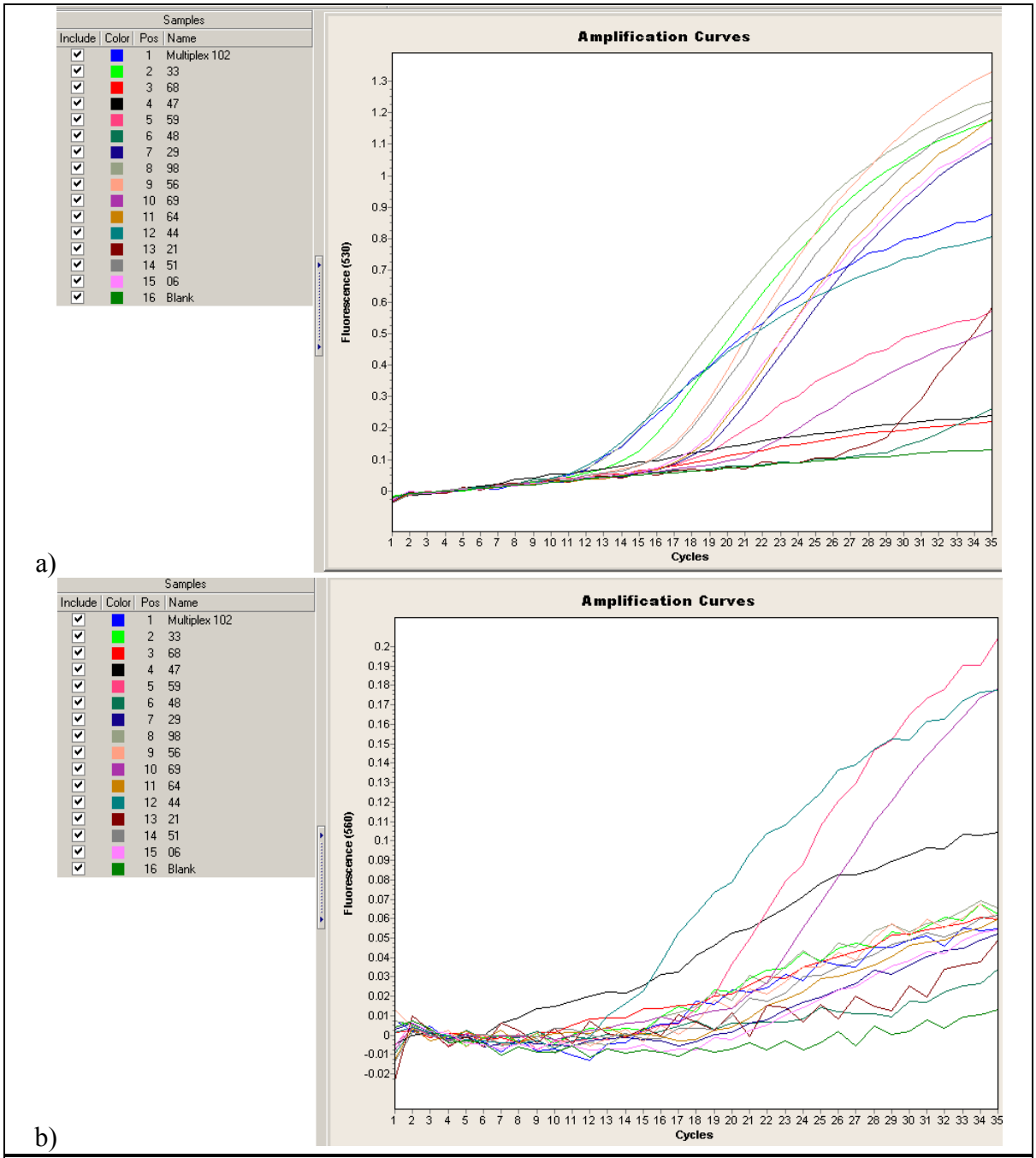


Figure 3.21: MGB multiplex reaction to detect K65R mutant and wild-type samples, with an annealing temperature of 62ºC. **a)** Wild-type samples were not detected (DR047, 048 and 068), and the rest successfully detected. **b)** 4 mutant samples were correctly detected (DR044, 047, 059, 069).

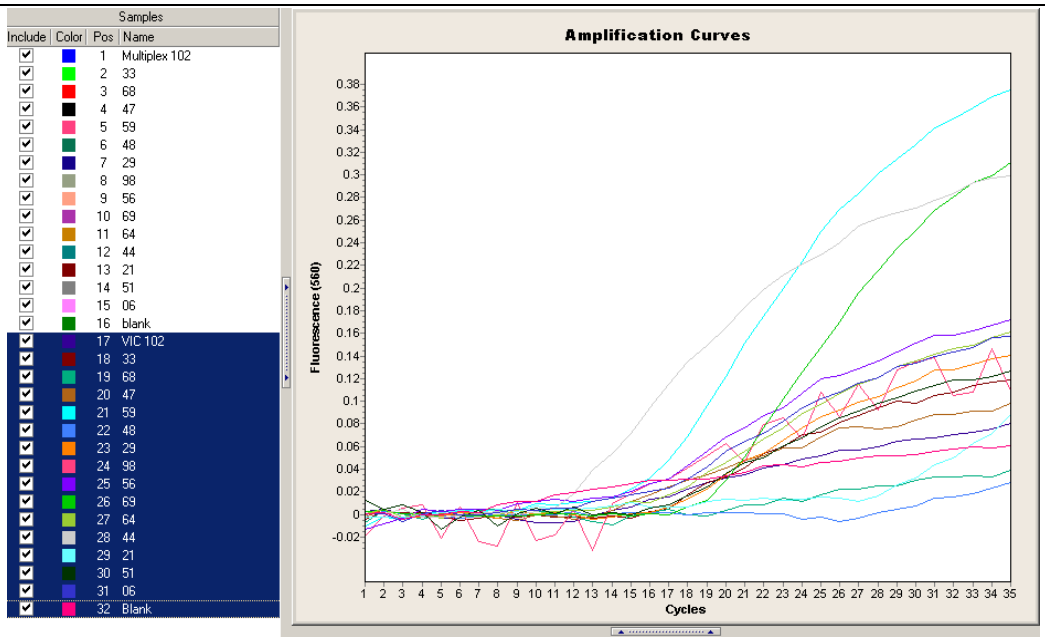


Figure 3.22: MGB single reaction detecting mutant samples with an annealing temperature of 60ºC. The VIC probe only detected 3 mutant samples (DR044, 059, 069).

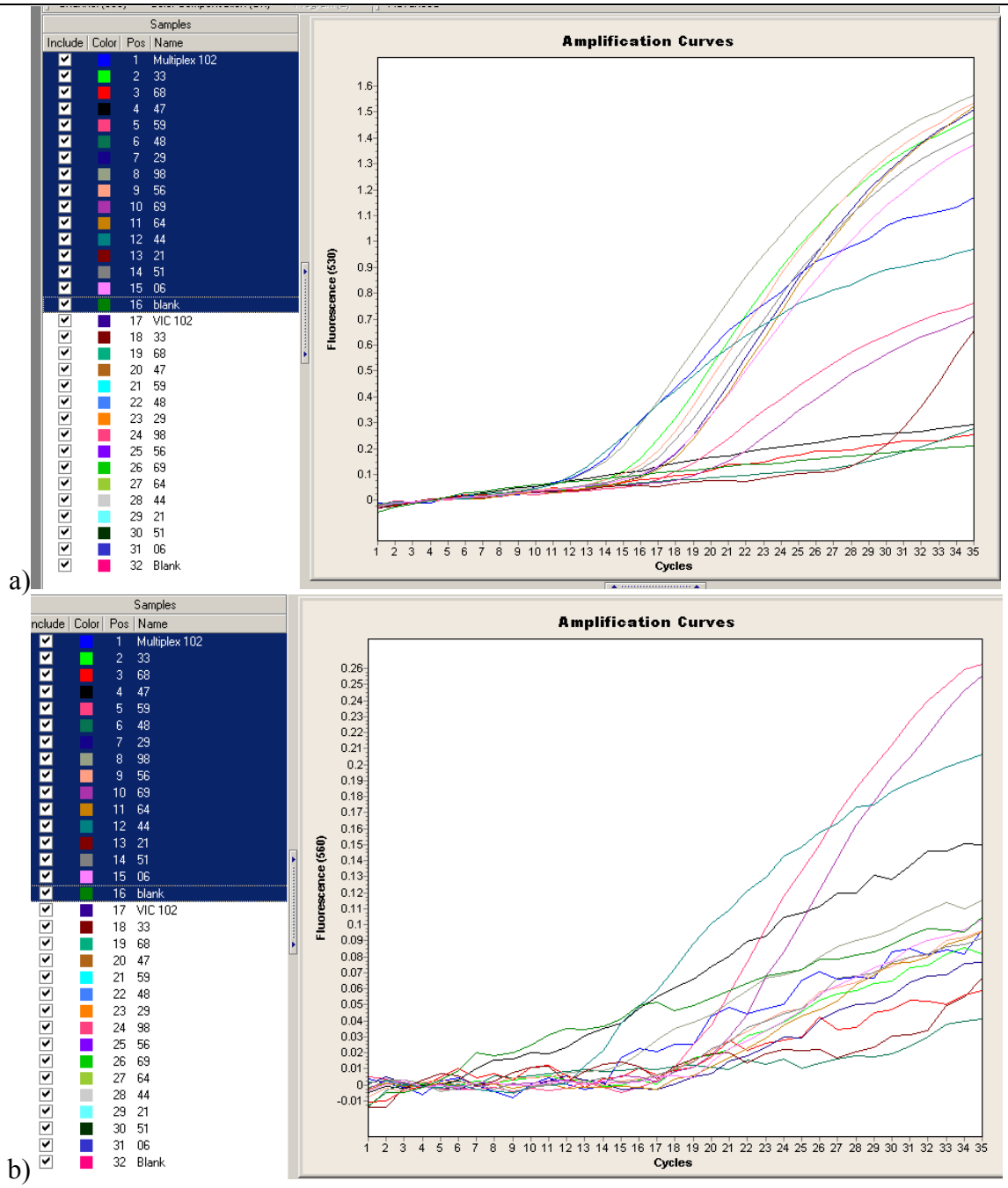


Figure 3.23: MGB multiplex reaction for K65R mutant and wild-type HIV-1 subtype C samples, with an annealing temperature of 60ºC. **a)** Wild-type samples detected was the same as with an annealing temperature of 62ºC and **b)** mutant HIV-1 subtype C samples with 4 correctly detected (DR044, 047, 059, 069).

The sequences of the samples used in the MGB assay were aligned (Figure 3.24) to determine the reason for indeterminate or poor MGB probe binding. Sequences correctly detected had 100% homology to the wild-type probe (DR006, 098, 033, 051, 056, 102) or mismatches 4 or less bases from the end of the probe (DR029). The two subtype B samples (DR021 and DR048) were only detected after cycle 29 (Figure 3.21), resulting from two mismatches which flank the K65R mutation.

All the samples containing the K65R mutation had lower fluorescent readings than the wild-type samples when detected with wild-type probe. The heterologous sample (DR044) had higher fluorescence than the 65R homologous samples (DR59 and DR69). These results clearly demonstrate that each sample had varying concentrations of mutant and wild-type species. The remaining two mutant samples (DR068 and DR047) were both not detected by the wild-type probe, as a result of a single mismatch at 6 and 7 bases (Figure 3.24), respectively from the 3'end of the probe. Thus, samples DR047 and DR068 were heterologous with both mutant and viral variants.

Four of the five mutant samples were successfully detected (Figure 3.21) with the mutant probe. The mutant sample (DR068) not detected had a single mismatch 6 bases from the 3'end of the mutant probe. Sample DR047 which was previously not detected by the wild-type probe because of a mismatch was detected by the mutant probe (Figure 3.24), which contained a degenerate base at this mismatch site. Sample DR047 presumably has a lower mutant viral population, as this sample's fluorescence was lower than the other 3 mutant samples (Figure 3.21).

Mutant Probe	TAT TTG CYA TAA AAA GGA A	Subtype
Wildtype Probe	AT TTG CCA TAA AAA AGA	
DR006	TAT TTG CCA TAA AAA AGA A	C
DR098	TAT TTG CCA TAA AAA AGA A	C
DR033	TAT TTG CCA TAA AAA AGA A	C
DR051	TAT TTG CCA TAA AAA AGA A	C
DR064	TAT TTG CCA TAA AAA AGA A	C
DR056	TAT TTG CCA TAA AAA AGA A	C
DR029	TAT TCG CCA TAA AAA AGA A	C
DR102	TAT TTG YCA TAA AAA AGA A	C
DR021	TAT TTG CCA TAA AGA AAA A	B
DR048	TAT TTG CCA TAA AGA ARA A	B
DR044	TAT TTG CCA TAA AAA RGA A	C
DR069	TAT TTG CCA TAA AAA GGA A	C
DR068	TAT TTG TCA TAA AAA RGA A	C
DR047	TAT TTG CTA TAA AAA RGA A	C
DR059	TAT TTG CCA TAA AAA GGA A	C

Figure 3.24: Alignment of samples detected with the MGB probes. The purple shaded letter of the mutant probe is the degenerate base; the blue shaded letters represent the mutation site; and the yellow shaded bases are the mismatches compared to the probe.

4. CHAPTER FOUR: DISCUSSION

The HIV/AIDS epidemic has reached epic proportions in South Africa, with over half a million AIDS patients being admitted into hospital in 2000, whom potentially required ARV treatment (African Action, 2003). The major concern as ARV therapy becomes more accessible, is the inevitable emergence of HIV-1 drug resistance. HIV-1 drug resistance results in treatment failure and the need for a change in treatment regimen. The potential transmission of resistant viral strains to treatment naïve individuals making future therapy ineffective is another grave consideration. In countries where ARV therapy has been available for several years, resistance transmission studies have indicated an underlying level of resistance of anywhere between 5-27% in drug naïve HIV-1 infected patients. (WHO/HIV/2003.10).

A recent mathematical model designed to determine the transmission of ARV drug resistant HIV strains from treated individuals in Africa (Blower, 2004), predicts that as ARV use increases there will be an exponential transmission of ARV drug resistant viruses. It is predicted that the accumulative effect over 5 years if 5% of the population are on ARVs, drug resistant HIV-1 will be transmitted to between 11 000-172 000 people. Similarly, if 10% of the population is being treated, 23 000-300 000 will be infected with drug resistant HIV-1 and finally if 25% of the population is treated, 19 000 to 855 000 people will be infected with drug resistant virus. These staggering numbers highlight the importance of an affordable alternative to the current gold standard ViroSeq for monitoring the emergence of drug resistance.

A limited amount of HIV drug resistance data is available in South Africa. Furthermore, data collected thus far is potentially skewed by the ad hoc fashion in which individuals accessed therapy prior to the national ARV roll-out program with financial constraints often leading to poor adherence and inadequate therapeutic regimens. These data therefore provides little knowledge of what level of resistance will result from the increased ARV usage in South Africa.

Appropriate national surveillance of the emergence of drug resistance thus needs to be conducted and local guidelines are currently under development to determine: the level of resistant virus circulating in an area; how resistance changes in the area in the presence of increased access to ARV therapy; to fully understand the determinants of resistance; how to minimize spread and ultimately be used to aid decisions on national level (WHO/HIV/2003.10) In addition, individual patient monitoring may need to be considered in certain scenarios in developing countries and on an individual basis.

Genotyping Assays such as ViroSeq are well suited to undertake resistance studies as the entire Protease and part of the Reverse Transcriptase genes are sequenced, allowing all drug resistant mutations including novel ones, to be detected simultaneously. Commercial genotyping assays such as ViroSeq are currently too costly (R3500/sample) to deploy on a national scale for individual patient monitoring in South Africa but may play a role in national surveillance programs. This assay has largely been validated in HIV-1 subtype B and is the assay of choice for patient monitoring in many developed countries. The assay was implemented locally to validate its usefulness in the South African scenario where the prevailing subtype is C. ViroSeq was validated initially using subtype B samples from

well characterized proficiency panels, analyzed in multiple centres and on multiple occasions in our laboratory. The results from the three VQA panels performed in our laboratory confirmed the accuracy, reproducibility and reliability, of the ViroSeq assay in our hands.

Furthermore, samples were analyzed to determine the effectiveness of this assay in HIV-1 subtype C. All HIV-1 subtype C samples were successfully amplified and sequenced using this assay. Of note is that one of the seven cycle sequencing primers, primer D, did not bind to subtype C amplicon. The failure of primer D binding did not alter the results of the assay, since primer A was able to generate the overlapping sequence. Moreover, the ViroSeq algorithm needs to be updated to contain HIV-1 subtype C specific mutations which confer resistance to several ARVs (Morris et al., 2003).

Our results are supported by several other studies from the region. Jagodzinski et al. (2003) showed the effectiveness of this assay in subtypes A through H. A Ugandan study indicated the success of the ViroSeq assay on various subtypes, namely, A, C, D and A/D recombinant HIV-1 (Mracna, et al., 2001). Furthermore, Eshleman et al., (2004b) were able to generate genotyping data for 99% of samples obtained from Uganda, Cameroon, South Africa, Argentina, Brazil and Thailand. The assay has also been used to monitor the emergence of drug resistance in a cohort of predominantly HIV-1 subtype C infected mothers receiving a single dose of NVP in South Africa (Pillay, et al., 2002).

For individual patient monitoring in resource poor settings like South Africa more affordable and accurate drug resistance monitoring assays are required. This study

therefore focused on developing and evaluating assays which would be reliable, rapid and affordable methodologies (Appendix E, Table E.1) compared to the ViroSeq assay. The point mutation assays developed and evaluated were chosen with these criteria in mind, and the results of these alternative genotyping assays (OLA, MGB, and RFLP) were compared to the gold standard ViroSeq genotyping system. Furthermore, the concept of investigating only certain mutations which are associated with particular drugs is well suited for the South African context where treatment regimens are well defined and standardized. The mutations for which the assays were designed and evaluated have been well described and shown to occur under specific ARV drug pressure in HIV-1 subtype B. The limited work conducted in South Africa to date support the choices made. There are however exceptions such as the V106M mutation which appears to be a novel HIV-1 subtype C mutation.

The three assays used for point mutation identification in this project all required an amplification step as the starting point. The detection steps of each of the separate assay approaches were evaluated for effectiveness and affordability and will be further evaluated longitudinally throughout an NIH funded program, the CIPRA program. This cohort will receive treatment regimens similar to those used in the ARV roll out program and will be used to generate important information around the appropriate timing and algorithm of such HIV drug resistance assays. OLA has been proposed as a cost effective, rapid and high volume test perfectly suited for resource poor environments; originally developed for HIV-1 subtype B (Beck, et al., 2002). OLA therefore needs to be evaluated on HIV-1 subtype C before being implemented to ensure that the viral diversity between subtypes does not negatively impact on the assay's performance.

The two other novel mutation detection approaches were developed. The first assay centred around the use of MGB modified probes in a real-time PCR assay format. Thus far the MGB probe design has not been used for diagnosis or monitoring in the HIV context. The MGB complex allows for an increase in annealing temperature thus allowing for a short probe of only 12 bases to be designed (Afonina, et al., 2002). In the context of the diverse HIV genome this allows for the least amount of mismatches between probe and target sequence. Further modification was made to the probe by incorporating a degenerate base to allow for sequence variation. In the second assay, a restriction enzyme was used. The restriction enzyme used in the RFLP assay was chosen as *Mae III*, which has a degenerate base at the recognition site for restriction, allowing both HIV-1 subtype C and B to be detected. All three detection assays were evaluated on HIV-1 subtype C samples from infected individuals on a variety of ARV combination therapies. The results were compared to mutation profiles obtained for the ViroSeq assay.

For the OLA, the amplification step was initially evaluated using the primers supplied in the OLA kit. As it was anticipated the sequence variation of the HIV virus between subtypes compromised primer binding resulting in no amplification of the HIV-1 subtype C samples. This was in contrast to previous studies where these primers had been used to successfully amplify DNA (Beck and Frankel, 2002) and RNA from HIV-1 subtype B samples (Beck, et al., 2002).

In order to improve the efficiency of the PCR amplification of either proviral DNA or viral RNA of HIV-1 subtype C samples, primers from the OLA kit were replaced with primers specifically designed for the reverse transcriptase region of the *pol* gene of HIV-1 subtype

C (ST1 and ST2; Figure 3.4). The subtype C specific primers (ST1 and ST2), were able to amplify fragments in a one-step PCR from samples with viral loads as low as 2450 copies/ml. Moreover, when second round primers (A-(35) and NE-1(35)) were introduced both HIV-1 subtype C and B (DNA control 8E5) samples were amplified. The ST1 and ST2 primers also amplified the subtype B plasmids, supplied in the OLA kit, thus eliminating the need for the extra set of OLA primers. However, it remains to be determined whether other HIV-1 subtype B samples will be amplified. Overall the one-step RT-PCR reaction was used to decrease turn around time and possible contamination. Thus, modifications to the prescribed OLA PCR amplification process allowed for a more robust amplification process with direct application to HIV-1 subtype C.

Similarly, the OLA detection probes in this assay were designed and evaluated on HIV-1 subtype B, with limited evaluation being conducted in non-B subtypes (Vega, et al., 2003). These probes were therefore evaluated for their ability to bind and recognize certain point mutations (K65R, K103N and Y181C) associated with the proposed drug regimens in HIV-1 subtype C samples. Overall evaluation resulted in poor detection of all mutations analysed in HIV-1 subtype C compared to sequencing genotyping results. The small number of HIV-1 subtype B samples evaluated, also demonstrated indeterminate binding of the probe compared to the mutations identified by genotyping (Table 2.2).

To allow for better detection of HIV-1 subtype C samples, modifications could be made to the detection probes and may provide the basis for future studies. The probe sequence can be changed to potentially decrease the number of mismatches between the probes and the subtype C samples and thus improve ligation efficiency (Table 4.1). In summary, the

K103N proposed probe changes would reduce mismatches to less than 4 per sample and thus potentially improve ligation efficiency. It is uncertain how to address the issue of samples containing mismatches at the ligation site, as for example the K103S polymorphism seen in the VQA samples. The inability of the OLA assay to efficiently discriminate between major mutations and polymorphisms in the ligation area has also been previously demonstrated by Beck et al., (2002) for codon V82A in the protease gene where there was an additional base change at the site of ligation. In conclusion, there was poor detection of K103N in both HIV-1 subtype B (22%) and C (44%) samples (Table 3.3).

Correct detection of the Y181C in HIV-1 subtype B occurred in 44% of the cases with poorer results for detection in HIV-1 subtype C (24%) samples (Table 3.3). The common probe for Y181C would require at least 3 nucleotide changes which should improve the binding of this probe, especially as one of the recommended changes is close to the ligation site (Table 4.1). However, the mutant/wild-type probes have several mismatches (e.g. M184V, Y188H/C and G190A) when compared to HIV-1 subtype C sequences that were not consistent across samples studied and there appears to be little that could be changed to improve binding of these probes.

There was poor detection of K65R in both HIV-1 subtype B (33%) and C (0%) samples (Table 3.3). The recommendation for improved probe binding to detect the K65R mutation would be to change 4 bases in the probes. Two of these changes flank the ligation site and may thus significantly influence ligation efficiency (Table 4.1).

The OLA assay in its current format is unable to effectively differentiate between major mutations conferring drug resistance or subtype related polymorphisms which may confer resistance in the ligation area. Beck et al., (2002) and Edelstein et al., (1998) both reported that their indeterminate probe binding was attributed to mismatches 3 bases from the ligation site or two or more base changes in the patient specimen in the region complimentary to either of the ligation probes.

The proposed changes outlined in Table 4.1 need further evaluation to ascertain whether detection would in fact be improved in the subtype C environment. The RT region is highly variable due to the error prone nature of RT during the viral replication cycle. It is thus extremely difficult to design a probe that will detect all HIV-1 sequences with 100% homology. Furthermore, the probes used to identify the resistance mutations in OLA are 40-50 bases long and the chances of mismatches occurring between the probe and complimentary sequence are thus extremely high. The cost of a single sample (assuming a full 96 well plate of 43 samples is run), including PCR, would cost approximately R158.80 per 6 mutations analyzed (Table E.1). This excludes the labour component which is significant since the assay has to be performed over several days (Table E.1). Further work is needed to establish whether probe changes suggested in this study (Figure 4.1) improve the detection of HIV-1 subtype C ARV drug resistance mutations.

Mutations associated with NNRTIs
K103N ACA TCC CGC AGG GTT AAA AAA GAA A*AAA TCA GTA ACA GTA CTG GAT GTG GGT ACA **C**CC **A**GC AGG GTT AAA AAA GAA A*AAA TCA GT**G** ACA GTA CTG GAT GTG GGT **Y181C** ACA AAA TCC AGA CAT AGT TAT CTA*TCA ATA CAT GGA TGA TTT GTA TGT A ACA AAA TCC AGA CAT AGT TAT CTA*TCA ATA **T**AT GGA TGA **C**TT **R**TA TGT A **Mutations associated with NNRTIs** **K65R** CTC CAG TAT TTG CCA TAA AGA A*RAA AGA CRG TAC TAA ATG GAG AA CTC CA**R** TAT TTG CCA TAA A**A**A A*RAA **G**GA CRG TAC TAA **G**TG GAG AA **Figure 4.1**: Nucleotide sequences of the present and proposed OLA probes. The first line for each mutation shows the wild-type (underlined) & common probe sequences, the asterisk the ligation site. Below each present OLA probe is the proposed subtype C probe, the bases bolded and underlined are those that would be changed to help in subtype C sample detection.

The second detection method, as described above, was designed using the endonuclease restriction enzyme *MaeIII* to discriminate between V106 and 106M/A/I in the PCR amplicons. HIV-1 subtype C has been found to have a polymorphism at codon 106 of the RT protein, which results in the novel signature mutation V106M under EFV pressure (Morris, et al., 2003). The V106M mutation was found to occur in 53% of patients treated with EFV, and in 5% of mothers treated with NVP to prevent vertical transmission of HIV-1 (Brenner, et al., 2003 and Morris, et al., 2003, Cane, et al., 2004). Most importantly, it causes cross-resistance to all 3 FDA approved NNRTIs (EFV, NVP and DLV; Brenner, et al., 2003). Furthermore, the emergence of V106M results in poor viral suppression and the subsequent development of the Y181C and Y188H/C mutations, which both cause a high level of resistance to NVP (Brenner, et al, 2003). V106A is a less common mutation in HIV-1 subtype C, but results in a 30-fold increase in resistance to NVP and a 2-5 fold increase in resistance to EFV and DLV (Shafer, 2004).

Results indicated that the RFLP assay correctly identified all V106A/M mutations and correlated 100% with genotypic data (Table 3.4). The assay is rapid and cost effective requiring a small amount of reagents and equipment. Furthermore, the results are easy to interpret. Theoretically this assay would also identify the extremely rare V106I mutation (recorded in only 0.1% of the population) which causes mild resistance when other NNRTI drug resistant mutations are present (Shafer, 2004).

The significant emergence of V106M in HIV-1 subtype C when exposed to EFV and a high dose of NVP, needs to be monitored since both NVP and EFV are drugs prescribed in South Africa's ART roll-out and in MTCT prevention programs. The early identification of this mutation in HAART patients could result in changes to the drug regimen, ensuring reduced emergence of additional HIV-1 drug resistant mutations, and prevent treatment failure. In the context of NVP used to prevent MTCT, the presence of V106M in the mother will render the infant susceptible to HIV-1 infection. This will have a direct impact on transmission rates and the success rate will decline. Furthermore, when MTCT prevention is ineffective, the resistant mutations can be transmitted to the baby (Loubster, et al., 2004). The transmission of a drug resistant HIV-1 strain to the infant would result in poor virological responses to subsequent prophylaxis. This assay would cost approximately R167 per sample and the total set up costs are R 43 316.25 (Table E.1), as the equipment require and labour is minimal. Furthermore, the easily interpreted results are obtained in 1.5 days.

The last detection step was evaluated as a proof of concept to determine the effectiveness of using the MGB probes for detection. The MGB probe proof of concept was evaluated

on the K65R mutation and potentially could be used to detect other mutations. The K65R mutation causes cross resistance to most NRTIs and is increasing in frequency in developed countries. The assay was run on both HIV-1 subtype C and B PCR amplicons. Interestingly, the HIV-1 subtype B plasmids (either 100% wildtype or mutant) could not be used as controls as the fluorescence was much lower than that of the HIV-1 subtype C samples. This is thought to be a result of poor probe and sample sequence affinity.

The multiplex reaction designed detected all 4 samples containing the K65R mutation (Figure 3.21) correctly and was found to be cheaper than performing two separate PCR reactions. Three of fifteen samples were not detected by the wild-type probes. This improved detection of codon 65 by the MGB assay, as compared to the OLA results was attributed to the design and shortened length of the MGB probes. The MGB probes were designed around the most conserved area around the K65R mutation. The probe was designed to bind downstream rather than upstream of the mutation to avoid binding at codons 67 and 69 where insertions and other mutations commonly occur. Sequence alignment and evaluation (Figure 3.24) indicated that mismatches close to the K65R mutation site resulted in no probe binding or fluorescence. Samples with lower levels of fluorescence or those for which fluorescence appeared later in the amplification step (Subtype B samples; Figure 3.21 and 3.23) were found to have mismatches further away from the point mutation site. Interestingly, end point fluorescence measurements were found to be inaccurate when reactions were multiplexed since relevant dyes chosen had overlapping fluorescence spectra. This particular assay could thus only be performed on an instrument such as the LightCycler that had a colour compensating ability. These could be detected using end point fluorescence on a simple fluorimeter when conducted in separate

reactions. This would however increase reagent costs because of the larger sample volume (minimum 50ul reaction) being required.

The OLA assay identified none of the HIV-1 subtype C samples at codon 65 correctly, most likely due to the many potential mismatches in its long probe sequence (50bp). However, the MGB probe's short length allowed for 100% detection of mutant viruses, and 80% detection of wild-type HIV-1 viruses. The MGB assay would therefore accurately genotype 80% of HIV-1 from patients at position 65 at a cost of R187.50 per mutation if multiplexed (Table E.1), resulting in only 20% of patients requiring population based sequencing at R3500 per sample.

Furthermore, this probe design can be adapted for the detection of other mutations. The small probe design would allow a number of other mutations, namely K103N and Y181C both associated with ARV resistance to be detected. The OLA detection of K65R, K103N and Y181C mutations could potentially be improved by the modification of probes to be more subtype specific or perhaps to modify them to an MGB type format.

Overall, results indicate that the real-time assay using MGB probes for monitoring of specific resistance mutations is an alternative to ViroSeq, as it is accurate, affordable (Table E.1), rapid and requires a minimal amount of hands on time. Moreover, the increased sensitivity of the MGB assay (5%) allows for smaller populations to be identified. This assay will allow for better information to be obtained as it has a higher sensitivity than the ViroSeq assay which only has a detection limit of 40%. This increased sensitivity makes this assay well suited for pooled surveillance studies. This would result

in a large amount of prevalence data for resistance mutations being acquired at a minimal cost.

All three of the point mutation assays investigated in this project have the potential to allow for informed decisions on resistance profiles in resource constrained settings. Each assay described in this study has a particular niche in ARV drug resistance testing in South Africa. ViroSeq is the gold standard assay and if resources allowed would currently be the assay of choice for both individual patient monitoring and surveillance. A modified subtype C specific OLA assay can have limited use in situations where, for example, patients are followed longitudinally and their baseline sequence data implies high probe ligation efficiency. The particular RFLP assay example described in this study for V106M could be used in patients receiving either EFV or NVP. The emergence of the novel V106M might also be used as a screen prior to implementation of therapy as this mutation results in a 50% chance of resistance to an entire class of ARV drugs. The MGB probe assay is a good method that can be used as a first line screen for the detection of a particular mutation.

In conclusion, a significant amount of work still needs to be conducted in the context of drug resistance in HIV subtype C in the South African environment. This will need to include the determination of all relevant mutations in response to different drug combinations, the level of resistance conferred by each mutation, and the relationship between different mutations. Once this information is available from local cohorts, assays and algorithms can be designed to support resistance monitoring in ARV treatment programs.

Appendix A: List of Solutions

A.1: 50x Tris Ethylenediaminetetra-acetic acid (TAE)

2mM Tris (MERCK, South Africa)

28.55ml glacial acetic acid (MERCK, South Africa)

2mM of 0.5M Ethylenediaminetetra-acetic acid (EDTA; SigmaGermany) pH8

Fill up with MilliQ water to 500ml

A.2: 2% Agarose Gel

2g Agarose Molecular Grade (Roche, Germany)

6mM of 10xEthidium bromide (Sigma, Germany)

Fill up with 1xTAE (Refer A.1) to 100ml

A.3: 10xLigase Buffer

200mM Tris (MERCK, South Africa), pH8

100mM $MgCl_2$ (Sigma, Germany)

10mM DTT (Rcohe, Germany)

A.4: Bovine Serum Albumin (BSA) Blocking Solution

0.5% BSA (Roche, USA) in Phosphate Buffer Saline (PBS, Roche, Germany)

A.5: 1xNaOH Wash

0.01N NaOH (Sigma, Germany)

0.05% Tween 20 (Roche, USA)

A.6: 10xTris Wash

1M Tris (MERCK, South Africa), pH7.5

1.5M NaCl (Sigma, Germany)

0.5% Tween 20 (Roche, USA)

A.7: Bromophenol Blue

0.25% Bromophenol Blue (Sigma, Germany)

0.1M EDTA (Sigma, USA)

50% Glycerol (MERCK, South Africa)

Fill up with MilliQ water 25ml

A.8: 3% Agarose Gel

3g Seakem® LE Agarose (Roche, Germany)

6mM of 10xEthidium bromide (Sigma, Germany)

Fill up with 1xTAE (Refer A.1) to 100ml

Appendix B: List of panel 4 (004g01-05) VQA a) sequences (FASTA) and b) mutation Lists (GT).

a)Sequences

004g01
Thu Oct 09 16:02:26 GMT+02:00 2003. 1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCATCGTCACAGTAAAAATAGCGGGACAACTAAAGGAAGCTATATTGGAT
ACAGGGGCAGATGAtACAGtaTTYGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAACTGATAGGGGGAATT
GGAGGTTTTGTCAAAGTAAGACAGTATGATCAGATACCCATAGAAATCTGCGGACATAAAACTATAGGTACAGTA
TTAATAGGACCTACACCTGCCAACATAATTGGAAGAGGTCTGATGACCCAGATTGGCTGCACTTTAAATTTCCCC
ATTAGTYCTATTGAAACTGTACCAGTAAAGTTAAAGCCAGGAATGGATGGCCCAAGAGTTAAACAATGGCCATTR
ACAGAAGAAAAAATAAAAGCATTAATGGaAATTTGTACAgAATTGGAAAAAGATGGAAAAATTTCAAAAATTGGG
CCTGAAAATCCATaCAATACTCCAGTATTTGYTATAAAAAAGAAAAACAGTACTAAATGGAGAAAAGTAGTAGAT
TTCAGRGAACTTAATAAAAGAACCCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCTGCAGGGATAAAA
ARGAACAAATCAGTATCAGTAATAGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGACTTTAGAAAG
TACACCGCATTTACTATACCCAGTACAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAG
GGATGGAAAGGATCACCAGCAataTTTCAATGTAGCATGACAAAAatcTTAGAaCcHTTTAGAAAacAAaATCCA
GACATAGtTaTCTATCAATAChTRGATGATTtGtatatAGgatctGaCTtagAAatAGGGAagcATagagCAAAA
aCaGaggaACTGagacAAtWTctGtGGAagtGGGGATTTtACACACCAGACAAAAAATATCAGAAAGAACCTCCA
TTCCTTTGGATGGGGTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAAGACAGC
TGGACTGTYAATGACATACAGAAGTTAGTGGGAAAATTRAATTGGGCAAGTCAAATTTATTCAGGGATTAAAGTA
AGGCAATTATGCAAACTCCTTAGGGGAACCAAATCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAGAGCTA
GAACtGGCAGAAAACAVKGAAATTCTAAAARAACCAGTACAtgGAGTGTAYTATGACCCAGCAAAAGACTTAATA
GCAGAAATACAGAAACAGGGGGAAGG

004g02
Tue Oct 28 14:08:24 GMT+02:00 2003. 1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCCGCATCRcAATAAAGGTAGGGGGGCAGCYAATAGAAGCTCTATTAGAT
ACAGGAGCAGATGATACAGTATTAGAAGACATAGATTTGCCAGGAAGATGGAAACCAAAATTGATAGGGGGAGTT
GGAGGTTTTGTCAAAGTAAAACAGTATGAGCAAGTACCCATAGAAATCTTAGGACATAARATTTTAGGTACAGTA
TTAGTAGGGCCTACACCTACCAACATAGTtGGAAGAAATGTGAtGGCTCAGATtGGTTGCACTTTAAATTTTCCC
ATTAGcTcTATTGACMCTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTG
ACAGAAGAAAAAATAAAAGCATTAGTAGAGATTTGTACATTTCTGGAACAGGAAGGAAAAATTTCAAARATWGGG
cCTGAAAATCCATacAATaCTCCAATATTTGCTATAAAGAAGAAAAACAGTGATCAATGGAGAAAATTAATGGAT
TTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGGATACCACATCCCGCAGGGTTAAAA
AAGAGCAAATCAGTAACAGTACTAgAcATAGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGAATTCAGGAAG
TATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATACCAGTACAATGTACTTCCACAG
GGATGGAAAGGATCACCAGCAATATTTCAAAGTAGCATGACAAGAATCTTAGAGCCTTTTAGAAAACAGAATCCA
GAAATARTTATCTAYCAATATGTGGATGATTTGTATGTAGCATCTGACTTAGAAATAAAGCAGCATAGAGCAAAA
ATAGAGGAACTGAGAGAATWTCTGTGGAAGTGGGGGTTTTACACACCAGACAAAAAGCATCAGAARGAACCTCCA
TTYCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAGGATAGC
TGGACTGTCAATGACATACAGAAGTTAGTGGGAAAATTGAATTGGGCAAGCCAGATTTATCCAGGGATTAAGGTA
AGGCAATTATGtAAACTCCTTAGGGGAGCCAAATCACtAACAGAAGtAGtACCACtAACAGCAGAAGCAGAACTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGARCCAGTACATGGAGYGTAYTATGACCCATCAAAAGACTTAATA
GCAGAAGTACAGAAGCAGGRGCAAGGC

004g03
Tue Oct 28 14:13:13 GMT+02:00 2003.1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCCGCGTCACAATAAAGGTAGGGGGGCAGCYAATAGAAGCTYTATTAGAT

ACAGGAGCAGATGATACAGTATTAGAAGACATAGATTTGCCAGGAAGATGGAARCCAAAATTGATAGGGGGAATT
GGAGGTTTTGTCAAAGTAAAACAGTATGAGCAAGTACCCATAGAAATCTTAGGACATAAGATTTTAGGTaCAGTA
TTAGTAGGGCCTACACCTACCAACATAATtGGAAGAAATCTGAtGACTCAGATtGGTTGCACTTTAAATTTTCCC
ATtAGCHcTATtGAMMCTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTG
ACAGAAGAAAAAATAAAAGCATTAGTAGAGATTTGTACATYtCTGGAACAGGAAGGAAAAATTTCaAAAATTGGG
CCTGAAAATCCATACAATACTCCAATATTTGCTATAAAGAAGAAAAACAGTGATAAATGGAGAAAATTAATGGAT
TTCAGAGAACTTAATAAGAGAACTCAAGAYTTCTGGGAAGTTCAATTAGGGATACCACATCCCGCAGGGTTAAAA
AAGAGCAAATCAGTAACAGTACTAGACATAGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGAATTCAGGAAG
TATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATACCAGTACAATGTACTTCCACAG
GGATGGAAAGGATCACCAGCAATATTTCAAAGTAGCATGACAAGAATCTTAGAGCCTTTtAgaAAACAGAATCCA
GAAATAGTTATCTATCAATAYGTGGATGATTTGTATGTAGCATCTGACTTAGAAATAAAGCAGCATAGAGCAAAA
ATAGAGGAACTGAGAGAATATCTGTGGAAGTGGGGGTTTTACACACCAGACAAAAAGCATCAGAAAGAACCTCCA
TTYCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAARGAYAGC
TGGACTGTCAATGACATACAGAARTTAGTGGGAAAATTGAATTGGGCAAGCCAGATTTATGCAGGGATTAAGGTA
AGGCAATTATGTAAACTCCTTAGGGGAGCCAAATCACTAACAGAAGTAGTACcACTAACAGCAGAAGCAgAACTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGAACCAGTACAtgGAGTGTAYTATGACCCATCAAAAGACTTAATA
GCAGAAGTRCAGAAGCAGGGGCAAGGC

004g04
Tue Oct 28 14:13:59 GMT+02:00 2003. 1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCTTAGTCACAGTAAGAATAGGGGGACAGCTAATAGAAGCCCTATTAGAC
ACAGGAGCAgATGATACAGTATTAGAAGACATGAGTTACCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTG
GAGGTTTTATCAAAGTAAGACAATATGATCAGATACCTATAGAAATTTGTGgAAAAAAGGCCATAGGTACAGTAT
TAGTGGGACCTACACCTGTCAACATAATTGGACGAAATATGTTGACTCAGATTGGTTGCACTTTAAATCTTCCAA
TTAGTCCTATTAAAACTGTGCCTGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAGCAATGGCCATTGA
CAGAAGAAAAAATAAAAGCATTAACAGacaTTTGTGCAGAGATGGAAAAGGAAGGAAAAATTTCAAAAATTGGGC
CTGAAAACCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGATAGTACCAAATGGAGAAAATTAGTAGATT
TCAGAGAACTCAATAAGAGAACTCAAGACTTCTGGGAGGTCCAATTAGGAATACCTCATCCCGCGGGATTAAAAG
AGAAAAAATCGGTAACAGTACTAGATGTGGGGGATGCATATTTTTCAGTTCCCTTAGATGAAGATTTTAGAAAGT
ATACTGCATTCACTATACCTAGTATAAATAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAAG
GATGGAAAGGATCACCAGCAATATTTCAGGCAAGCATGACRAAAATCTTAGAgCCCTTCAgAACAAAAAATCCAG
AGATGGTGATCTACCAATATATGGATGATTTATATGTAGGATCTGACTTAGAGATAGGGCAGCATAGAGCAAAAA
TAGAGCAGTTGAGAGACCATCTACTGAAATGGGGATTTACCACACCAGACAAAAAACATCAGAAGGAaCCCCCAT
TTCTTTGGATGGGATATGAACTCCATCCTGACAAATGGaCAGTCCAGCCTATACAGCTGCCAGAAAAGGACAGCT
GGaCTGTCAATGATATaCAGAAATTAGTGGGAAAAcTAAATTGGGCAAGTCAGATCTATGCAGGAATTAGAGTAA
AGCAATTGTGtAAACTCCTCAGGGGAGCCAAAGCACTAACAGATAtAGTAacactgAcTGAGGAAGCAGAATTAG
AATTGGCAGAGAATAGGGAAATTCTAAAAAAACCTGTACATGGAGTATATTATGACCCRACAAAAGACTTAGTAG
CAGAAATACAGAAACAAGGGCAAGAC

004g05
Tue Oct 28 14:15:25 GMT+02:00 2003. 1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCCGCATCRCAATAAAGGTAGGGGGGCAGCYAATAGAAGCTCTATTAGAT
ACAGGAGCAGATGATACAGTATTAGAAGACATAGATTTGCCAGGAAGATGGAAACCAAAATTGATAGGGGGAGTT
GGAGGTTTTGTCAAAGTAAAACAGTATGAGCAAGTACCCATAGAAATCTTAGGACATAARATTTTAGGTACAGTA
TTAGTAGGGCCTACACCTACCAACATAGTtGGAAGAAATGTGAtGGCTCAGATtGGTTGCACTTTAAATTTTCCC
ATTAGCtCTATTGACMCTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTG
ACAGAAGAAAAAATAAAAGCATTAGTAGAGAtTTGTACATTTCTGGAACAGGAAGGAAAAATTTCAAARATWGGG
CCTGAAAATCCATACAATACTCCAATATTTGCTATAAAGAAGAAAAACAGTGATCAATGGAGAAAATTAATGGAT
TTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGGATACCACATCCCGCAGGGTTAAAA
AAGAGCAAATCAGTAACAGTACTAGACATAGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGAATTCAGGAAG
TATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATACCAGTACAATGTACTTCCACAG
GGATGGAAAGGATCACCAGCAATATTTCAAAGTAGYATGACAAGAATCTTAGAgCCTTTTAGAAAACAGAATCCA
GAAATARTTATCTAYCAATATGTGGATGATTTGTATGTAGCATCTGACTTAGAAATAAAGCAGCATAGAGCAAAA

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ATAGAGGAACTGAGAGAATWTCTGTGGAAGTGGGGGTTTTACACACCAGACAAAAAGCATCAGAARGAACCTCCA
TTYCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAGGATAGC
TGGACTGTCAATGACATACAGAARTTAGTGGGAAAATTGAATTGGGCAAGCCAGATTTATCCAGGGATTAAGGTA
AGGCAATTATGTAAACTCCTTAGGGGAGCCAAATCACTAACAGAAGTAGTACCACTAACAGCAGAAGCAGAACTA
GAACtGGCAGAAAACAGGGAAATTCTAAAAGARCCAGTACATGGAGYGTAYTAtGACCCATCAAAAGACTTAATA
GCAGAAGTACAGAAGCAGGRGCAAGGC
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b) Mutation Lists

```
004g01
Protease
L     10    I
L     33    F
M     46    L
I     54    V
L     63    P
A     71    T
V     77    I
V     82    A
L     90    M
V     3     I
I     13    V
G     16    A
L     23    I
N     88    G
004g01
Reverse Transcriptase
M     41    L
E     44    D
A     62    V
D     67    N
L     74    V
L     100   I
K     103   N
M     184   I
L     210   W
T     215   Y
P     4     S
K     20    R
V     35    M
K     102   R
T     107   S
L     109   I
E     122   K
I     135   T
S     162   C
M     184   L
V     189   I
Q     197   K
T     200   A
I     202   T
H     208   Y
```

H	208	F
R	211	K
L	214	F
H	221	Y
P	272	S
A	288	S
I	293	V
R	307	K
R	307	N
R	307	T
R	307	S
E	312	K
S	322	A
Q	334	E
G	335	-

004g02
Protease

L	10	R
M	36	I
M	46	L
50	V	0
I	54	V
L	63	P
A	71	I
V	82	T
L	90	M
V	3	I
V	11	I
T	12	A
I	15	V
L	19	P
K	20	I
E	35	D
S	37	D
R	57	K
D	60	E
I	62	V
C	67	L
I	72	L
I	85	V
L	89	V
T	91	A

004g02
Reverse Transcriptase

M	41	L
D	67	N
T	69	D
M	184	V
G	190	A
L	210	W
T	215	Y
G	333	E

P	4	S
E	6	D
T	7	P
E	40	F
K	43	Q
V	60	I
K	70	Q
V	75	M
K	103	S
V	111	I
E	122	K
D	123	E
K	166	R
D	177	E
V	179	I
G	196	K
T	200	A
Q	207	E
H	208	Y
H	208	F
R	211	K
L	214	F
T	286	A
A	288	S
I	293	V
E	297	A
V	317	A
I	329	V

004g03
Protease

L	10	R
M	36	I
M	46	L
I	54	V
L	63	P
A	71	I
V	82	T
L	90	M
V	3	I
I	15	V
L	19	P
K	20	I
E	35	D
S	37	D
R	57	K
D	60	E
I	62	V
C	67	L
I	72	L

004g03
Reverse Transcriptase

M	41	L

D	67	N
T	69	D
M	184	V
G	190	A
L	210	W
T	215	Y
P	4	T
P	4	S
E	6	D
T	7	P
E	40	S
E	40	F
K	43	Q
V	60	I
V	75	M
K	103	S
V	111	I
E	122	K
D	123	E
K	166	R
D	177	E
G	196	K
T	200	A
Q	207	E
H	208	Y
R	211	K
L	214	F
P	272	A
T	286	A
A	288	S
I	293	V
E	297	A
I	329	V

004g04
Protease

L	63	P
V	3	I
I	13	V
K	14	R
K	20	I
E	35	D
S	37	-
H	69	K
L	89	M
F	99	L

004g04
Reverse Transcriptase

E	6	K
V	35	T
E	36	D
T	39	A
K	102	E

S	162	A
K	173	T
Q	174	K
D	177	E
I	178	M
T	200	A
E	204	Q
Q	207	D
R	211	K
L	214	F
V	245	Q
P	272	A
K	275	R
R	277	K
T	286	A
E	291	D
V	292	I
I	293	V
P	294	T
E	312	K
S	322	T
I	326	V
G	335	D

004g05
Protease

L	63	P
V	3	I
I	13	V
K	14	R
K	20	I
E	35	D
S	37	-
H	69	K
L	89	M
F	99	L

004g05
Reverse Transcriptase

E	6	K
V	35	T
E	36	D
T	39	A
K	102	E
S	162	A
K	173	T
Q	174	K
D	177	E
I	178	M
T	200	A
E	204	Q
Q	207	D
R	211	K
L	214	F

V	245	Q
P	272	A
K	275	R
R	277	K
T	286	A
E	291	D
V	292	I
I	293	V
P	294	T
E	312	K
S	322	T
I	326	V
G	335	D

Appendix C: List of panel 5 (005g01-05) VQA a) sequences (FASTA) and b) mutation Lists (GT).

a) Sequences

005g01
Wed Oct 29 11:24:47 GMT+02:00 2003. 1302bases.
GCAGCTAATAGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGACATAGATTTGCCAGGAAGATG
GAARCCAAAATTGATAGGGGGAATTGGAGGTTTTGTCAAAGTAAAACAGTATGAGCAAGTACCCATAGAAATCTT
AGGACATAAGATTTTAGGTACAGTATTAGTAGGGCCTACACCTACCAACATAATtGGAAGAAATCTGAtGACTCA
GATtGGTTGCACTTTAAATTTTCCCATtAGcycTATtGAMMCTGTACCAGTAAAATTAAAGCCAGGAATGGATGG
CCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGaGATTTGTACATYTCTGGAACA
GGAAGGAAAAATTTCaAAAATTGGgcCTGAAAATCCATaCAAtaCTCCAATATTTGCTATAAAGAAGAAAAACAG
TGATAAATGGAGAAAATTAATGGATTTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGG
GATACCACATCCCGCAGGGTTAAAAAAGAGCAAATCAGTAACAGTACTAgAcATAGGTGATGCATATTTTTCAGT
TCCCTTAGATAAAGAATTCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAG
ATACCAGTACAATGTACTTCCACAGGGATGGAAAGGATCACCAGCAATATTTCAAAGTAGCATGACAAGAATCTT
AGAgCCTTTTAgAAAACAGAATCCAGAAATAGTTATCTATCAATAyGTGGATGATTTGTATGTAGCATCTGACTT
AGAAATAAAGCAGCATAGAGCAAAAATAGAGGAACTGAGAGAATATCTGTGGAAGTGGGGGTTTTACACACCAGA
CAAAAAGCATCAGAAAGAACCTCCATTYCTTTGGATGGGYTATGAACTCCATCCTGATAAATGGACAGTACAGCC
TATAGTGCTGCCAGAAAARGAYAGCTGGACTGTCAATGACATACAGAARTTAGTGGGAAAATTGAATTGGGCAAG
CCAGATTTATGCAGGGATTAAGGTAAGGCAATTATGTAAACTCCTTAGGGGAGCCAAATCACTAACAGAAGtAGT
ACCACTAACAGCAGAAGCAGAACTAGAACtGGCAGAAAACAGGGAAATTCtAAAAGAACCAGTACAtGGAGTGTA
YTATGACCCATCAAAAGACTTAATAGCAGAAGTACAGAAGCAGGGGCAAGGC

005g02
Wed Oct 29 11:26:55 GMT+02:00 2003. 1302bases.
AACGACCCATCGTCACAGTAAAAATAGCGGGACAACTAAAGGAAGCTATATTGGATACAGGGGCAGATGATACAG
TATTYGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAACTGATAGGGGGAATTGGAGGTTTTGTCAAAGTAA
GACAGTATGATCAGATACCCATAGAAATCTGCGGACATAAAACTATAGGTACAGTATTAATAGGACCTACACCTG
CCAACATAATTGGAAGAGGTCTGATGACCCAGATTGGCTGCACTTTAAATTTCCCCATTAGTYCTATTGAAACTG
TACCAGTAAAGTTAAAGCCAGGAATGGATGGCCCAAGAGTTAAACAATGGCCATTRACAGAAGAAAAAATAAAAG
CATTAATGGAAATTTGTACAGAATTGGAAAAAGATGGAAAAATTTCAAAAATTGGGCCTGAAAATCCATACAATA
CTCCAGTATTTGYTATAAAAAAGAAAAACAGTACTAAATGGAGAAAAGTAGTAGATTTCAGRGAACTTAATAAAA
GAACCCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCTGCAGGGATAAAAARGAACAAATCAGTATCAG
TAATAGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGACTTTAGAAAGTACACCGCATTTACTATAC
CCAGTACAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAG
CAATATTTCAATGTAGCATGACAAAAATCTTAGAACCATTTAGAAAACAAAATCCAGACATAGTTATCTATCAAT
ACMTRGATGATTTGTATATAGGATCTGACTTAGAAATAGGGAAGCATAGAGCAAAAACAGAGGAACTGAGACAAT
ATCTGTGGAAGTGGGGATTTTACACACCAGACAAAAAAYATCAGAAAGAACCTCCATTCCTTTGGATGGGGTATG
AACTCCATCCTGATAARTGGACAGTACAGCCTATAGTGCTGCCAGAAAAAGACAGCTGGACTGTYAATGACATAC
AGAAGHTAGTGGGAAAATTRAATTGGGCAAGTCAAATTTATTCAGGGATTAAAGTAAGGCAATTATGCAAACTCC
TTAGGGGAACCAAATCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAGAGCTAGAACTGGCAGAAAACAGGG
AAATTCTAAAAGAACCAGTACATGGAGTGTATTATGACCCAGCAAAAGACTTAATAGCAGAAATACAGAAACAGG
GGGAAGGT

005g03
Thu Oct 23 16:11:55 GMT+02:00 2003. 1248bases.
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TATTAGAAGACATAAATTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAA

GACAGTATGATCAGGTACCCATAGAAATcTGTGGACATAAAGTTATARGTWCAGTATTAGTAGGACCTACACCTG
TCAACATAATtGGAAGAAATCTGAtGACTCAGMTtGGTTGCAMTTTAAATTTTCCCATtAGtCCTATtGAAACTG
TACCAGTAAAATTRAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTGACAGAAGAaAAAATAAAAG
CATTAACAGAAATTTGTACAGAAATGGARAAGGAAGGRAAAATTTCAAAAATTGGGCCTGAAAATCCATATAATA
CTCCAGTATTTGCCATAAAGAAAAAGGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGAKAACTTAATAAGA
KAACTCAAGAYtTcTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAaAAAATCAGTAACAG
TAcTRGATGTGGGTGATGCATATtTCTCAGTTCCcTTAGATGAAGAHTTCAGGAAGTATACTGCATTCACCATAC
CTAGTATAAAYAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTCCCACAGGGATGGAAAGGATCACCAG
CAATATTCCAAAGTAGCATGACAAAAATcTTAGAGCCTTTTAGAAAAAAGAaTCCAGACATRGTTATCTATCAAT
ACGTGGATGATTTGTATGTAGGATCTGATTTRGAAATAGGGCAGCAYAGAGCAAAAATAGAGGAACTGAGACAAC
ATCTGTTGAAATGGGGGTTTACCACACCAGACAARAAACAYCAGAAAGAACCTCCATTCCTTTGGATGGGTTATG
AACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGTTGCCAGACAAAGAAAGCTGGACTGTCAATGACATAC
AGAAGTTAGTGGGAAAATTGAATTGGGCYAGTCAGATTTATGCAGGAATTAAAGTAAAGCAATTATGTAAACTCC
TTAGGGGAACCAAAGCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAGAGCTAGAATTGGCAGAAAACAGGG
AAATTYTAAAAGARCCAGTACATGGGGTG

005g04
Tue Oct 28 12:07:54 GMT+02:00 2003. 1302bases.
AACGacCCCTCGTCACAaTAAAGATAGGGGGGCAGCTAATAGAAGCTCTATTAGATACAGGAGCAGATGATACAG
TATTAGAAGACATAAATTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAA
GACAGTATGATCAGGTACCCATAGAAATCTGTGGACATAAAGTTATArGTwcaGtATTAGTAGGACCTACACcTG
TCAACATAATTGGAAGAAaTcTGAtGAcTCAGMTtGGTTGYActtTAAAtttTCCCAtTAgTCcTAttGAAAcTG
TACcAgTAAAAtTRAAGCcAGGAATGGATGGCCcaAAAgtTAaacAATGGCcAtTGAcAGAAGAAAAAATAaAAG
cAtTAAcAGAAAttTGTAcAGAAATGGARAAGGAAGGRAAAAttTcAAAAAtTGGGccTGAaaATccATATAATA
cTCcAGTAttTGccATAAAGAAAAAGGAcAGTAcTAAATGGAGAAAAtTAgTAGAttTcAGAgAActTAATAAGA
gAAcTCAAGAytTcTGGGAAgTTCAAtTAGGAATACCACATCCCGCAgGGTTAAAAAAgAaAAAATCAGTAACAG
TACTAGATGTGGGTGATGCATATTTCTCAgTTCCCTTAgAtGAagAcTTCAgGAagTATACTgCATTCACCATAC
CTAgTATAAAYAATGAgAcACCAgGGATTAGATATCAGTACAATgTGCTCCCACAggGATggAaaggATCACCAg
CAATATTCCaAagTAGCATGACAAAAATCTTAgAgCCTTTTAgAaAaaagAaTCCAgACATRgTTATCTATCAAT
ACGTGGATGATTTGTATGTAGGATCTGATTTaGAAATAGGGCAGCAYAgAgCAAAAATAGAgGAaCTGAgACAAC
ATCTGTTGAAATGGGGGTTTACCACACCAGACAAAAAACAYCAGAAAGAACCTCCATTCCTTTGGATGGGTTATG
AACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGTTGCCAGACAAAGAAAGCTGGACTGTCAATGACATAC
AGAAGTTAGTGGGAAAATTRAATTGGGCTAGTCAGATTTATGCAGGAATTAAAGTAAAGCAATTATGTAAACTCC
TTAGGGGAACCAAAGCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAGAGCTAGAATTGGCAGAAAACAGGG
AAATTYTAAAAGAGCCAGTACATGGGGTGTATTAtGacCcaaCAAAAGACTTAATAGCAGAGATACAGAAGCAGG
GGGAAGGT

005g05
Tue Oct 28 12:11:49 GMT+02:00 2003. 1302bases.
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TATTAGAAGACATAAATTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAA
GACAGTATGATCAGGTACCCATAGAAATCTGTGGACATAAAGTTATARGTWCAGTATTAGTAGGACCTACACCTG
TCAACATAATtGGAAGAAATCTGAtGACTCAGMTtGGTTGYACTTTAAATTTTCCCATTAGtCCTATtGAAACTG
TACCAGTAAAATTRAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAG
CATTAACAGAAATTTGTACAGAAATGGARAAGGAAGGRAAAATTTCAAAAATTGGGCCTGAAAATCCATATAATA
CTCCAGTATTTGCCATAAAGAAAAAGGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGAGAACTTAATAAGA
GAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCAGTAACAG
TACTAGATGTGGGTGATGCATATTTCTCAGTTCCCTTAGATGAAGAcTTCAGGAAGTATACTGCATTCACCATAC
CTAGTATAAAYAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTCCCACAGGGATGGAAaGGATCACCAG
CAATATTCCAAAGTAGCATGACAAAAATcTTAGagCCTTTTAgAAAAAAGAATCCAGACATRGTTATCTATCAAT
AcGTGGATGATTTGTATGTAGGATCTGATTTRGAAATAGGGCAGCAYAGAGCAAAAATAGAGGAACTGAGACAAC
ATCTGTTGAAATGGGGGTTTACCACACCAGACAAAAAACAyCAGAAAGAACCTCCATTCCTTTGGATGGGTTATG
AACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGTTGCCAGACAAAGaAAGCTGGACTGTCAATGACATAC

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AGAAGTTAGTGGGAAAATTRAATTGGGCYAGTCAGATTTATGCAGGAATTAAAGTAAAGCAATTATGTAAACTCC
TTAGGGGAACCAAAGCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAGAGCTAGAATtGGCAGAAAACAGGG
AAATTYTAAAAGAGCCAGTACAtgGGGTGTATTATGACCCAACAAAAGACTTAATAGCAGAGATACAGAAGCAGG
GGGAAGGT
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b) Mutation Lists

```
005g01
Protease
L     10    R
M     36    I
M     46    L
I     54    V
L     63    P
A     71    I
V     82    T
L     90    M
V     3     I
I     15    V
K     20    I
E     35    D
S     37    D
R     57    K
D     60    E
I     62    V
C     67    L
I     72    L
005g01
Reverse Transcriptase
M     41    L
D     67    N
T     69    D
M     184   V
G     190   A
L     210   W
T     215   Y
P     4     S
E     6     D
T     7     P
E     40    S
E     40    F
K     43    Q
V     60    I
V     75    M
K     103   S
V     111   I
E     122   K
D     123   E
K     166   R
D     177   E
G     196   K
```

T	200	A
Q	207	E
H	208	Y
R	211	K
L	214	F
P	272	A
T	286	A
A	288	S
I	293	V
E	297	A
I	329	V

005g02
Protease

L	10	I
L	33	F
M	46	L
I	54	V
L	63	P
A	71	T
V	77	I
V	82	A
L	90	M
V	3	I
I	13	V
G	16	A
L	23	I
N	88	G

005g02
Reverse Transcriptase

M	41	L
E	44	D
A	62	V
D	67	N
L	74	V
L	100	I
K	103	N
M	184	I
L	210	W
T	215	Y
P	4	S
K	20	R
V	35	M
K	102	R
T	107	S
L	109	I
E	122	K
I	135	T
S	162	C
M	184	L
V	189	I
Q	197	K
T	200	A

I	202	T
H	208	Y
R	211	K
L	214	F
H	221	Y
L	260	I
P	272	S
A	288	S
I	293	V
S	322	A
Q	334	E

005g03
Protease

M	36	I
L	63	P
A	71	V
G	73	S
L	90	M
V	3	I
T	4	N
K	20	I
E	35	D
S	37	N
I	62	V
T	74	S
I	93	L
T	96	N

005g03
Reverse Transcriptase

M	184	V
V	35	T
E	79	.
R	83	I
D	123	E
Q	174	K
I	178	M
T	200	A
R	211	K
L	214	F
E	248	D
D	250	E
P	272	A
R	277	K
I	293	V

005g04
Protease

M	36	I
L	63	P
A	71	V
G	73	S

L 90 M
V 3 I
K 20 I
E 35 D
S 37 N
I 62 V
T 74 S
I 93 L

005g04
Reverse Transcriptase
M 184 V
V 35 T
Q 174 K
I 178 M
T 200 A
R 211 K
L 214 F
E 248 D
D 250 E
P 272 A
R 277 K
I 293 V
S 322 T
Q 334 E

005g05
Protease
M 36 I
L 63 P
A 71 V
G 73 S
L 90 M
V 3 I
K 20 I
E 35 D
S 37 N
I 62 V
T 74 S
I 93 L

005g05
Reverse Transcriptase
M 184 V
V 35 T
Q 174 K
I 178 M
T 200 A
R 211 K
L 214 F
E 248 D
D 250 E
P 272 A
R 277 K
I 293 V

S	322	T
Q	334	E

Appendix D: List of panel 6 (006g01-05) VQA a) sequences (FASTA) and b) mutation Lists (GT).

a) Sequences

006g01
Fri Aug 20 15:08:34 GMT+02:00 2004. 1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCCGCATCRCAATAAAGGTAGGGGGGCAGCYAATAGAAGCTCTATTAGAT
ACAGgAgCAGATGATAcAgtATTAGAAGACATAGATTTGCCAGGAAGATGGAAACCAAAATTGATAGGGGGAGTT
GGAGGTTTTGTCAAAGTAAAACAGTATGAGCAAGTACCCATAGAAATCTTAGGACATAARATTTTAGGTACAGTA
TTAGTAGGGCCTacaccTaccAACATAgTtGGAAgAaATGTGATGGCTCAGATTGGTTGCACTTTAAATTTTCCC
ATTAGCTCTATTGACMCTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTG
ACAGAAGAaAAAATAAAAGCATTAGTAGAgATTTGTACATTTCTGGAACAGGAAGGAAAAATTTCAAARATWGGG
CCTGAAAATCCATACAATACTCCAATATTTGCTATAAAGAAGAaAAACAGTGATCAATGGAGAAAATTAATGGAT
TTCAGAgAACTTAATAAGAgAaCTCAAGAcTTCTGGGAAgTTCAATTAGGGATACCACATCCCGCAgGGTTAAAA
AAGAKCAAATCAGTAACAGTACTAGACATAGGTGATGCATATTtTTCAGTTCCCTTAGATAAAgAWTTCAGGAWG
TATACTGCATTTACCATACCTAGTATAAACAATGAGAcACCAGGGATTAGATACCAGTACAATGTACTTCCACAg
GGATGGAAAgGATCACCAGCAATATTTCAAAGTAGCATGACAARAATCTTAGAgCCTTTTAGAAAACAGAATCCA
GAAATAATTATCTAYCAATATGTggATGATTTGTATGTAGCaTCTGACTTAGAAATAAAGCAGCATAGAGCAAAA
ATAGAgGAACTGAGAgAaTWTCTGTGGAAgTGGGGGTTTTACACACCAGACAAAAAGCATCAGAARGAACCTCCA
TTYCTTTGGATGGGTTATGAACTCCATCCTGATAAATGgACAGTACAGCCTATAGTGCTGCCAGAAAAGGATAGC
TGGACTGTCAATGACATACAGAARTTAGTGGGAAAATTGAATTGGGCAAGCCAGATTTATCCAGGGATTAAGGTA
AGGCAATTATGTAAACTCCTTAGGGGAGCCAAATCACTAACAGAAGTAGTaCCACTAACAGCAGAAGcAgAACTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGARCCAGTACATGGAGYGTACTATGACCCATCAAAAGACTTAATA
GCAGAAGTACAgAAGCAGGgGCAAGGC

006g02
Tue Aug 10 15:37:06 GMT+02:00 2004. 1298 bases.
CCTCAAATCACTCTTTGGCAACGACCCCGCATCRCAATAAAGGTAGGGGGGCAGCYAATAGAAGCTCTATTAGAT
ACAgGAGCAGATGATACAGTATTAGAAGACATAGATTTGCCAGGAAGATGGAAACCAAAATTGATAGGGGGAGTT
gGAGGTTTtGtcaaaGTAAAACAGTATGAGCAAGTACCCATAGAAATCTTAGGACATAARATTTTAGGTACAGTA
TTAgTaggGCCTACACCtaCCAACATAGTtGGAAGAAATGTGATGGCTCAGATTGGTTGCACTTTAAATTTTCCC
ATTAGCTCTATTGACMCTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTG
ACAGAAGAAAAAATAAAAGCATTAGTAGAGATTTGTACATTTCTGGAACAGGAAGGAAAAATTTCAAARATWGgg
CCTGAAAATCCATaCAATaCtCcAATATTTGCTATAAAGAAGAAAAACAGTGATCAATGGAGAAAATTaATGGAT
TTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGGATACCACATCCCGCAGGGTTAAAA
AAGAGCAAATCAGTAACAGTACTAGACATAGGTGATGCATATTTTtCAGTTCCCTTAGATAAAGAATTCAGGAAG
TATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATACCAGTACAATGTACTTCCACAG
GGATGGAAAGGATCACCAGCAATATTTCAAAGTAGYATGACAAGAATCTTAGAGCCTTTTAGAAAACAGAATCCA
GAAATAATTATCTAYCAATAYGTGGATGATTTGTaTGTAGCATCTGACTTAGAAATAAAGCAGCATAGAGCAAAA
ATAGAGGAACTGAGAGAATWTCTGTGGAAGTGGGGGTTTTACACACCAGACAAAAAGCATCAGAARGAACCTCCA
TTYCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAGGATAGC
TGGACTGTCAATGACATACAGAARTTAGTGGGAAAATTGAATTGGGCAAGCCAGATTTATCCAGGGATTAAGGTA
AGGCAATTATGTAAACTCCTTAGGGGAGCCAAATCACTAACAGAAGTAGTACCACTAACAGCAGAAGCAGAACTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGARCCAGTACATGGAGtGTAYTATGACCCATCAAAAGACTTAATA
GCAGAAGTACAGAAGCAGGRGCA

006g03
Tue Aug 10 15:53:04 GMT+02:00 2004. 1267 bases.
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ACAGGGGCAGATGATaCAGTATTYGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAACTGATAGGGGGAATT
GGAGGTTTTGTCAAAGTAAGACAGTATGATCAGATACCCATAGAAATCTGCGGACATAAAACTATAGGTACAGTA

TTAATAGGACCTACACCTGcCAACATAATTGGAAGAGgTCTGATGACCCAGATTGGCTGCACTTTAAATTTCCCC
ATTAGTYCTATTGAAACTGTACCAGTAAAGTTAAAGCCAGGAATGGATGGCCCAAGAGTTAAACAATGGCCATTR
ACAGAAGAAAAAATAAAAGCATTAATGGAAATTTGTACAGAATTGGAAAAAGATGGAAAAATTTCAAAAATTGGG
CCTGAAAATCCATACAATACTCCAGTATTTGYTATAAAAAAGAAAAACAGTACTAAATGGAGAAAAGTAGTAGAT
TTCAGRGAACTTAATAAAAGAACCCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCTGCAGGGATAAAA
ARGAACAAATCAGTATCAGTAATAGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGACTTTAGAAAG
TAyACCGCATTTACTATACCyAGTACAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAG
GGATGGAAAGGATCACCAGCAATATTTCAATGTAGCATGACAAAAATCTTAGAACCATTTAGAAAACAAAATCCA
GACATAGTTATCTATCAATACCTRGATGATTTGTATATAGGATCTGACTTAGAAATAGGGAAGCATAGAGCAAAA
ACAGAGGAACTGAGACAATATCTGTGGAAGTGGGGATTTTACACACCAGACAAAAAAYATCAGAAAGAACCTCCA
TTCCTTTGGATGGGGTATGAACTCCATCCTGATAARTGGACAGTACAGCCTATAGTGCTGCCAGAAAAAGACAGC
TGGACTGTYAATGACATACAGAAGTTAGTGGGAAAATTRAATTGGGCAAGTCAAATTTATTCAGGGATTAAAGTA
AGGCAATTATGCAAACTCCTTAGGGGAACCAAATCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAGAGCTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGAACCAGTACATGGAGTGTATTATGACCCAGCAAAAG

006g04
Tue Aug 10 16:06:04 GMT+02:00 2004. 1302 bases.
CCTCAAATCACTCTTTGGCAACGACCCCGCATCRCAaTAaaGGTAGGGGGGCAGCYAATAGAAGCTCTATTAGAT
ACAGGAGCAGATGATACAGTATTAGAAGACATAGATTTGCCAGGAAGATGGAAACCAAAATTGATAGGGGGagTT
GGAGGtTTTGtcAAAGtAAAACAGTATGAGCAAGTACCCATAGAAATCTTAGGACATAARATTTTAGGTACAGTA
TTAGTAGGGCCTACACCTACCAACATAGTtGGAAGAAATGTGAtGGCTCAGATtGGTTGCACTTTAAATTTTCCC
ATTAGctctATTGACaCTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAAACAATGGCCATTG
ACAGAAGAAAAAATaAaAGCATTAGTAgAGATTTGTACATTTCTGGAACAGGAAGGAAAAATTTCAAARATWGGG
CCTGAAAATCCATACAATACTCCAATATTTGCTATAAAGAAGAAAAACAGTGATCAATGGAGAAAATTAATGGAT
TTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGGATACCACATCCCGCAGGGTTAAAA
AAGAGCAAATCAGTAACAGTACTAGACATAGGTGATGCATATTTTTCAGTTCCCTTAGATAAAGAATTCAGGAAG
TATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATACCAGTACAATGTACTTCCACAG
GGATGGAAAGGATCACCAGCAATATTTCAAAGTAGCATGACAAGAATCTTAGAGCCTTTTAGAAAACAGAATCCA
GAAATAATTATCTAYCAATATGTGGATGATTTGTATGTAGCATCTGACTTAGAAATAAAGCAGCATAGAGCAAAA
ATAGAGGAACTGAGAGAATWTCTGTGGAAGTGGGGGTTTTACACACCAGACAAAAAGCATCAGAARGAACCTCCA
TTYCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAGGATAGC
TGGACTGTCAATGACATACAGAARTTAGTGGGAAAATTGAATTGGGCAAGCCAGATTTATCCAGGGATTAAGGTA
AGGCAATTATGTAAACTCCTTAGGGGAGCCAAATCACTAACAGAAGTAGTACCACTAACAGCAGAAGCAGAACTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGARCCAGTACATGGAGYGTAYTATGACCCATCAAAAGACTTAATA
GCAGAAGTACAGAAGCAGGRGCAAGGC

006g05
Tue Aug 10 16:01:00 GMT+02:00 2004. 1302 bases.
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GGAGGTTTTGtCAAAGTAAGACAGTATGATCAGATACCCATAGAAATCTGCGGACATAAAACcATAGGTACAGTA
TTAATAGGACCTACACCTGcCAACATAATtGGAAGAGGTCTGAtGACCCAGATtGGCTGCACTTTAAATTTCCCC
ATTAGTCCTATTGAAACTGTACCAGTAAAGTTAAAGCCAGGAATGGATGGCCCAAGAGTTAAACAATGGCCATTG
ACAGAAGAAAAAATAAAAGCATTAATGGAAATTTGTACAGAATTGGAAAAAGATGGAAAAATTTcAAAAaTTGGg
CCTGAAAATCCATACAATACTCCAGTATTTGCTATAAAAAAGAAAAACAGTACTAAATGGAGAAAAGTAGTAGAT
TTCAGGGAACTTAATAAAAGAACCCAaGACTTcTGGGAAGTTCAATTAGGAATACCACATCCTGCAGGGATAAAA
AAGAACAAATCAGTATCAGTAaTAGATGtGGGTGATGCATATTTTtcAGTtCCCTTAGATAAAgACTTTAgAAAG
TACACCGCATTTACTATACCCAGTACAAACAATGAGACACCAGGGATTAGATATcAGTaCAATGTGCTTcCACAG
GGATGGAAAGGATCACCAGCAATATTTCAATGTagCATGACAAAAaTCTTAGACCCTTttAgAAAACAAAATCCA
GACaTAGtTtTcTATcAATacATGGATGATTTGtaTATaGGATCTGACTTAgAAaTAGGGAAGCATaGAgcAaaA
ACAgAGGAACTGaGGCAaTTtCTGTGGAAgtGGgGATTTTACACACCAGACAAAAAACATcAGAAAGAACCTCCA
TTCCTTTGGATGGGGTATGAACTCCATCCTGATAAGTGGACAGTACAGCCTATAGKGCTGCCAGAAAAAGACAGC
TGGACTGTTAATGACATACAGAAGTTAGTGGGAAAATTGAATtGGGCAAGTCAAATtTATTCAGGGATTAAAGTA

AGGCAATTATGCAAACTCCTTAGGGGAACCAAATCACTAACAGAAGTAGTACCACTAACAGAAGAAGCAgAGCTA
GAACTGGCAGAAAACAGGGAAATTCTAAAAGAACCAGTACATGGAGTGTATTATGACCCAGCAAAAGACTTAATA
GCAGAAATACAGAAACAGGGGGAAGGT

b) Mutation Lists

006g01
Protease

L	10	R
M	36	I
M	46	L
I	50	V
I	54	V
L	63	P
V	82	T
L	90	M
V	3	I
V	11	I
T	12	A
I	15	V
L	19	P
K	20	I
E	35	D
S	37	D
R	57	K
D	60	E
I	62	V
C	67	L
A	71	I
I	72	L
I	85	V
L	89	V
T	91	A

006g01
Reverse Transcriptase

M	41	L
D	67	N
T	69	D
V	75	M
K	103	S
M	184	V
G	190	A
L	210	W
T	215	Y
P	4	S
E	6	D
T	7	P
E	40	F
K	43	Q
V	60	I
K	70	Q
K	103	I
V	111	I

E	122	K
D	123	E
K	126	M
K	166	R
D	177	E
V	179	I
G	196	K
T	200	A
Q	207	E
H	208	Y
H	208	F
R	211	K
L	214	F
T	286	A
A	288	S
I	293	V
E	297	A
V	317	A
I	329	V

006g02
Protease

L	10	R
M	36	I
M	46	L
I	50	V
I	54	V
L	63	P
V	82	T
L	90	M
V	3	I
V	11	I
T	12	A
I	15	V
L	19	P
K	20	I
E	35	D
S	37	D
R	57	K
D	60	E
I	62	V
C	67	L
A	71	I
I	72	L
I	85	V
L	89	V
T	91	A

006g02
Reverse Transcriptase

M	41	L
D	67	N
T	69	D
V	75	M

K	103	S
M	184	V
G	190	A
L	210	W
T	215	Y
G	333	E
P	4	S
E	6	D
T	7	P
E	40	F
K	43	Q
V	60	I
K	70	Q
V	111	I
E	122	K
D	123	E
K	166	R
D	177	E
V	179	I
G	196	K
T	200	A
Q	207	E
H	208	Y
H	208	F
R	211	K
L	214	F
T	286	A
A	288	S
I	293	V
E	297	A
I	329	V

006g03
Protease

L	10	I
L	33	F
M	46	L
I	54	V
L	63	P
A	71	T
V	77	I
V	82	A
L	90	M
V	3	I
I	13	V
G	16	A
L	23	I
N	88	G

006g03
Reverse Transcriptase

M	41	L
E	44	D
A	62	V

D	67	N
L	74	V
L	100	I
K	103	N
L	210	W
T	215	Y
P	4	S
K	20	R
V	35	M
K	102	R
T	107	S
L	109	I
E	122	K
I	135	T
S	162	C
M	184	L
V	189	I
Q	197	K
T	200	A
I	202	T
H	208	Y
R	211	K
L	214	F
H	221	Y
P	272	S
A	288	S
I	293	V
S	322	A

006g04
Protease

L	10	R
M	36	I
M	46	L
I	50	V
I	54	V
L	63	P
V	82	T
L	90	M
V	3	I
V	11	I
T	12	A
I	15	V
L	19	P
K	20	I
E	35	D
S	37	D
R	57	K
D	60	E
I	62	V
C	67	L
A	71	I
I	72	L

I 85 V
L 89 V
T 91 A
006g04
Reverse Transcriptase
M 41 L
D 67 N
T 69 D
V 75 M
K 103 S
M 184 V
G 190 A
L 210 W
T 215 Y
G 333 E
P 4 S
E 6 D
E 40 F
K 43 Q
V 60 I
K 70 Q
V 111 I
E 122 K
D 123 E
K 166 R
D 177 E
V 179 I
G 196 K
T 200 A
Q 207 E
H 208 Y
H 208 F
R 211 K
L 214 F
T 286 A
A 288 S
I 293 V
E 297 A
V 317 A
I 329 V

006g05
Protease
L 10 I
L 10 V
L 10 F
L 33 F
M 46 L
I 54 V
L 63 P
A 71 T
V 77 I
V 82 A

L	90	M
V	3	I
P	9	N
P	9	K
P	9	T
P	9	S
P	9	R
P	9	I
P	9	M
P	9	H
P	9	Q
P	9	L
P	9	D
P	9	E
P	9	A
P	9	G
P	9	V
P	9	Y
P	9	.
P	9	C
P	9	W
P	9	F
L	10	K
L	10	N
L	10	T
L	10	M
L	10	Q
L	10	H
L	10	P
L	10	E
L	10	D
L	10	A
L	10	.
L	10	Y
L	10	S
V	11	K
V	11	N
V	11	R
V	11	S
V	11	I
V	11	Q
V	11	H
V	11	L
V	11	E
V	11	D
V	11	G
V	11	.
V	11	Y
V	11	C
V	11	F
T	12	R
T	12	S
T	12	I

T 12 M
T 12 P
T 12 L
T 12 A
T 12 G
T 12 V
I 13 Q
I 13 L
I 13 E
I 13 V
I 13 .
K 14 R
K 14 E
K 14 G
I 15 V
G 16 A
L 23 I
N 88 G
006g05
Reverse Transcriptase
M 41 L
E 44 D
D 67 N
L 74 V
L 100 I
K 103 N
L 210 W
T 215 Y
K 20 R
V 35 M
T 107 S
L 109 I
E 122 K
I 135 T
S 162 C
E 169 D
I 180 F
V 189 I
Q 197 K
T 200 A
I 202 T
H 208 F
R 211 K
L 214 F
V 245 G
P 272 S
A 288 S
I 293 V
S 322 A
Q 334 E

Appendix E: Comparison of Costing for OLA, RFLP and MGB

Table E1: Comparison of the cost of OLA, RFLP and MGB. This price is based on the cost per sample run with the appropriate controls. In addition, cost of final algorithm will be determined by the number of reactions that are performed. (Costing on 23 February 2005, with R5.92 to $1.)

	ASSAYS		
	OLA	**RFLP**	**MGB**
RNA Extraction	R 42.00	R 42.00	R 42.00
RT-PCR	R 41.10	R 41.10	R 41.10
Nested PCR	R 17.66	R 17.66	N/A
GEL Analysis	R 20	R 20	R 20
Detection Step	R 26.88	R 35	R 47.35
Consumables (Tips, Gloves, Tubes, etc.)	R 11.24	R 11.24	R 9.74
Total Cost for Reagents and consumables	**R 158.80**	**R 167**	**R 160.19**
Labour (Excluded)	**R 750 (12hrs)**	**R 281.25 (4.5hrs)**	**R 187.50 (3hrs)**
Total set up costs(Reagents, Labour and Instruments)	**R 116 908.80**	**R 43 316.25**	**R 440 347.69**
Turn Around time	**3 Days**	**1.5 Days**	**1 Day**

Appendix F:

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Evaluation of an oligonucleotide ligation assay for detection of mutations in HIV-1 subtype C individuals who have high level resistance to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors

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Abstract

The oligonucleotide ligation assay (OLA) has been proposed as an affordable alternative to sequence-based HIV-1 drug resistance testing in resource poor settings. The aim was to evaluate OLA for detecting mutations K103N, Y181C, K65R, Q151M, M184V and T215Y/F in subtype C.

Forty-four subtype C and 8 subtype B HIV-1 positive individuals were analysed using the ViroSeq™ HIV-1 genotyping assay (Applied Biosystems, Foster City, CA). A one-step RT-PCR and nested PCR were performed using subtype B specific primers from the OLA kit (NIH AIDS Research and Reference Reagent Program). Seventy-eight subtype C sequences were used to design subtype C specific primers. Ligation and detection steps were followed according to OLA kit protocol. For codons, K103N, Y181C, K65R, Q151M, M184V and T215Y/F, four or more mismatches compared to the probe or mismatches less than four bases from the ligation site were not tolerated. Results revealed accurate identification of mutations in 2/10, 4/9 3/9, 6/7, 2/7 and 6/7 VQA samples and 5/20, 4/17 0/20, 18/24, 5/24 and 13/24 subtype C positive individuals, respectively.

It was concluded that the probes and primers in the NIH reference kit would need modification to optimize detection of mutations in subtype C individuals.

Keywords: HIV-1; Subtype C; Drug resistance; OLA

1. Introduction

The development of human immunodeficiency virus type 1 (HIV-1) drug resistance can be attributed to several factors including poor drug adherence, inefficient drug regimens and development of viral resistance. The importance of monitoring viral resistance has been outlined by several panels of experts, namely, the International AIDS society, USA (IAS, USA) panel and the Euro Guidelines Group (EGG). These panels recommend that resistance testing is performed in the following cases: primary infection; chronic infection; treatment failure; pregnancy; post exposure; treatment interruption. Most of these panels recommend that both phenotypic and genotypic resistance assays be performed. Such recommendations are based on retrospective and prospective clinical trials, which demonstrate the utility of resistance testing. (Baxter et al., 2000; Durant et al., 1999).

South Africa has an estimated 5 million adults and children living with HIV/AIDS (WHO, 2004 http://www.who.int/3by5/coverage/en) and annual national antenatal

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surveillance prevalence has risen steadily over a decade with an HIV prevalence of 26.5% in 2002 (South African HIV/AIDS, 2005 Statistics http://www.avert.org/safricastats.htm). By 2005, it is expected that HIV/AIDS will cost South Africa 1% of the gross domestic product and account for a staggering 75% of its health budget (South Africa Department of Health, 2005: http://www.doh.gov.za/docs/pr/2003/pr0703.html). These numbers make conventional genotyping methodologies at costs ranging between US$ 250 and US$ 475 per assay simply unaffordable for individual patient management of virological failure.

Recently more affordable genotypic resistance testing strategies such as the oligonucleotide ligation assay (OLA) have been proposed as potential, sensitive and cost effective alternatives to conventional sequencing approaches (Edelstein et al., 1998; Beck et al., 2002). Oligonucleotide ligation assays have been described as a method to detect known sequence variants in a standardised ELISA plate format. In this assay, either amplified RNA or DNA serve as templates for allele-specific ligation reactions using three oligonucleotide probes (common, wild-type and mutant). Following hybridisation, the common probe binds adjacent to the differentially labelled wild-type or mutant probe and hybridised probes are then ligated using a ligase enzyme resulting in the joining two probes either common to wild-type or common to mutant. Ligation products are then captured on a solid support and stringent washes are performed to remove unbound oligonucleotides (Samiotaki et al., 1994). The colour signals generated from the two probes can be read photometrically on a conventional ELISA plate reader.

The OLA assay is currently available for evaluation through the National Institutes of Health (NIH, 2005) AIDS Research and Reference Reagent Program (www.aidsreagent.org) and have kits for the detection of resistance mutations associated with three classes of antiretroviral agents: the protease inhibitors; nucleoside reverse transcriptase inhibitors (NRTI); non-nucleoside reverse transcriptase inhibitors (NNRTI). Several mutations were selected for evaluation based on the treatment regimens to be implemented in the proposed anti-retroviral therapy (ARV) rollout program to be initiated in South Africa. Since the regimens are likely to include drugs from the reverse transcriptase inhibitor classes and be protease inhibitor-sparing regimens, the following kits were selected: (1) the NRTI OLA reagent kit containing 6 primers, 13 codon-specific oligonucleotides and control genomic DNA mutations K65R, Q151M, M184V and T215Y/F are evaluated in this assay, (2) the NNRTI OLA reagent kit containing 6 primers, 6 codon specific oligonucleotides and control genomic DNA. From this kit, the mutations K103N and Y181C were studied.

Previous publications have reported on the performance of this assay in HIV-1 subtype B individuals (Beck et al., 2002; Edelstein et al., 1998). This paper includes preliminary data on the performance of the assay in subtype C infected individuals on ARV treatment.

2. Materials and methods

2.1. Specimen collection

Stored plasma and PCR products from 44 HIV-1 positive individuals on anti-retroviral therapy programs were selected randomly for use in this study. These samples had all been submitted for routine genotyping evaluation using Viroseq™ HIV-1 Genotyping version 2.5 (Applied Biosystems, Foster City, CA) and selections were made based on (1) the drug resistance mutations identified and (2) classification as HIV-1 subtype C. An additional 10 samples were included for analysis from the NIH Virology Quality Assurance Program (VQA): 8 subtype B (004g01-03, 004g05 and 005g01-05) and 1 recombinant virus CRF02_AG (004g04). Both sets of samples were analysed using the commercial Viroseq™ genotyping assay and the OLA assay as per manufacturer's instructions.

2.2. RNA extraction and optimisation of RT-PCR step

Briefly, RNA extraction was performed using 200 μl of sample plasma with the High Pure Viral Nucleic Acid Kit (Roche, Germany). Fifty microliters of diluted RNA was used for the RT-PCR step which was conducted using OLA primers in a one-step PCR reaction and from RNA using the Viroseq™ RT-PCR module and a nested reaction using primers designed specifically for subtype C samples, ST1 and ST2 (shown below). Different permutations were used for this RT-PCR step in an attempt to reduce total PCR time by introducing a one-step PCR; and to establish the optimal methodology for amplification of subtype C samples.

In strategy 1 (from RNA using OLA primers in a nested PCR), the OLA protocol was modified to a one-step RT-PCR assay to simplify the process and reduce time taken to conduct the assay. The extracted RNA was amplified using the Titan One Tube RT-PCR System (Roche, Penzberg, Germany) followed by a nested PCR reaction. Both PCR reactions performed used the primers supplied in the OLA kits (NIH AIDS research and reference reagent program cat number: 7644 and 7645). The one-step Titan kit synthesizes double-stranded DNA directly from RNA. This one-step reaction was carried out as described by the kit protocol, using a total reaction volume of 50 μl, containing 10 μl of RNA and 0.4 μM of primer PRA and RTA (supplied in the kit received from NIH AIDS research and reference reagent program). The amplification protocol chosen included an annealing temperature of 50 °C for the non-specific cycling step (10 cycles) and 55 °C for the specific cycling step (40 cycles). The nested PCR was performed on the product obtained as described in the kit protocol (Beck and Frenkel, 2002). A 1019 bp fragment was obtained spanning from HIV-1 *gag* to codon 237 of the reverse transcriptase gene.

In strategy 2 (from RNA using the ViroSeq™ RT-PCR module and a nested reaction using ST1 and ST2 primers): RNA was amplified using the ViroSeq™ RT-PCR Module

version 2 (Applied Biosystems, Foster City, CA), and a nested PCR performed using primers specifically designed for subtype C. The product obtained from the ViroSeq RT-PCR was genotyped. A nested PCR was performed with the ST primers to ensure all products detected were of the same size. The primers were designed by aligning 78 HIV-1 subtype C sequences (courtesy Prof. L. Morris, NICD), to amplify up a 543 bp region encompassing all the major reverse transcriptase mutations conferring resistance. These primers were also used to amplify the plasmids supplied in the OLA kits. The sequences of the primers were as follows: ST1 3′-AAA ATT GGG CCT GAA AAT CC-5′ forward primer and ST2 3′-ATC CAA AGA AAT GGG GGT TC-5′ reverse primer. The nested PCR reaction was carried out in a 40 μl reaction volume, containing 4 μl of PCR product, 2.5 mM dNTP's (AB gene, Surrey, United Kingdom), 2 pmol of primers ST1 and ST2, 1.5 mM $MgCl_2$, 1× PCR buffer and 0.05 U/μl Taq DNA polymerase (Fermentas, Lithuania). The optimised cycling conditions consisted of an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final elongation step of 72 °C for 7 min. All PCR products obtained were visualized on a 2% agarose gel following electrophoresis and ethidium bromide staining to ensure successful amplification.

2.3. Oligonucleotide ligation assay

The OLA detection procedure occurs by ligating a codon-specific probe to the PCR product generated. After ligation is complete the product is captured on a streptavidin-coated plate (StreptaWell, Roche, Mannheim, Germany) and incubated with antibodies anti-Digoxigenin-POD, (Roche, Mannheim, Germany) and anti-fluorescein-alkaline phosphatase (Roche, Penzberg, Germany). The presence of a mutation at that codon is identified by a pink colour change as a result of the addition of GIBCOBRI® amplifier and substrate (GIBCOBRI®, Paisley, Scotland). The wild-type is identified by a colour change of blue to yellow using 3, 3′,5,5′-tetramethylbenzidine (Roche, Indianapolis, IN, USA) and H_2SO_4 (MERCK, South Africa). The procedure and reaction conditions for detection were thus carried out exactly as described in the protocol by Beck and Frenkel (2002) provided with the NIH reference reagent kits. During optimisation of this assay it was noted that if the PCR product added to the ligation mix was increased to 5 μl (from 2 μl), better identification of the controls (plasmids and control DNA supplied in the kit) and samples was observed. The assay was thus evaluated using 5 μl of PCR product and each assay repeated three times to assess for reproducibility and verification of results.

The following mutations were evaluated: NRTI kit: K65R, Q151M, M184V, T215Y/F and from the NNRTI kit: K103N and Y181C. All samples and controls provided were tested in duplicate during the conduct of each assay. Optical density (OD) readings were obtained from both the wild-type and mutant detection steps, and the reading subtracted from an average of the four blanks run with each assay. The OD readings obtained were plotted on a scatter plot (mutation versus wild-type). A cut-off for both mutant and wild-type was determined using the mean OD for either wild-type or mutant plasmid ±2.5S.D. (Beck et al., 2002). To control for variation between assays both wild-type and mutant controls for each codon tested were included in every run.

2.4. Sequencing

VQA samples were sequenced following the ViroSeq™ protocol. Briefly RNA was isolated from plasma, and amplified in a two-step RT-PCR. The PCR product was purified and analysed on a 2% agarose gel. The PCR product was diluted if required and cycle sequencing was performed using seven primers provided in the kit. The cycle sequencing product was purified using the sodium acetate method. The samples were denatured and loaded onto the 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were then analysed on version 2.5 of the Viroseq™ software and subtyping was performed using the Stanford database.

3. Results

3.1. Results of PCR assays

The products obtained from PCR reactions performed using the primers supplied in the OLA kit and the subtype C specific primers were compared. It was demonstrated that the OLA primers did not amplify the subtype C samples as efficiently as the subtype C primers, ST1 and ST2.

3.2. Detection of resistance mutations in the PCR product

Both subtype B and subtype C samples were evaluated, the subtype B samples were analysed to determine OLA efficiency and the subtype C samples to evaluate OLA on subtype C. Each sample was run in duplicate in three separate assays. In all the assay runs, plasmid controls supplied in the OLA kits were included. In all the assays reported in this evaluation, plasmids were correctly identified as either wild-type or mutant. The cut-offs for both the wild-type and mutants were obtained from optical density readings by determining the standard deviation of the mutant and wild-type blanks and subtracting these from 2.5 (Beck et al., 2002).

3.2.1. VQA samples

A total of nine VQA samples were used, including eight subtype B (004g01-03, 004g05 and 005g01-05) and one recombinant virus CRF02_AG (004g04) supplied by the Virology Quality Assurance Laboratory (NIH). The following codons were evaluated: K103N, Y181C of the NNRTI kit and K65R, Q151M, M184V and T215F/Y of the NRTI kit. The results were as follows: 2/9, 4/9 for the NNRTI codons and

Table 1
Summary of codons analysed using the OLA NNRTI and NRTI kits

Codon	K103N			Y181C			K65R		
	Plasmid	VQA	Subtype C	Plasmid	VQA	Subtype C	Plasmid	VQA	Subtype C
Total samples	6	9	20	6	9	17	6	9	19
No. of wild-type	5	3	9	5	9	14	5	9	15
No. of mutant	1	6	11	1	0	3	1	0	4
Total no. correctly identified	6	2	5	6	4	4	6	3	0
Percentage correctly identified	100	22	25	100	44	24	100	33	0

Codon	Q151M			M184V			T215Y/F		
	Plasmid	VQA	Subtype C	Plasmid	VQA	Subtype C	Plasmid	VQA	Subtype C
Total samples	6	7	24	6	7	24	6	7	24
No. of wild-type	1	7	23	1	5	15	5	3	11
No. of mutant	5	0	1	5	2	9	1	4	13
Total no. correctly identified	6	6	18	6	2	5	6	6	12
Percentage correctly identified	100	86	72	100	29	21	100	86	42

The table indicates how all controls (plasmids) supplied with the kits were correctly identified. This is unlike both the VQA subtype B samples and subtype C patient samples. This reduced identification was attributed to sequence variation when the sequences were analysed.

3/9, 6/7, 2/7, 6/7 for the NRTI codons were detected correctly (Table 1).

For codon 103, of the nine samples, three were wild-type and 6 were mutant as determined by genotyping. However, only two of these samples (005g03 and 005g05) were correctly identified by OLA (Table 1). Both of these samples had wild-type sequences that showed 100% homology to the probe (Fig. 1a). The third wild-type sequence (004g04) had five mismatches compared to the probe. Of the six mutant sequences, four had the unusual K103S mutation (004g02, 004g03, 004g05 and 005g01) as well as three other mismatches that probably contributed to either no binding or low binding to both wild-type and mutant probes. Both K103N mutant sequences (004g01 and 005g02) had four mismatches compared to the probe.

Similarly, for codon 181, all nine sample were wild-type as determined by genotyping. However, only four of these samples (004g03, 005g02, 005g03 and 005g05) were correctly identified by OLA (Table 1), three (004g03, 005g03 and 005g05) of which had wild-type sequences that had three to four mismatches at a significant distance from the ligation site. The remaining wild-type sequence correctly detected (005g02) had 100% homology to the probe (Fig. 1b). Of the non-detected wild-type sequences, sample 004g01 had three mismatches compared to the probe, probably resulting in no binding, or insufficient binding of this probe resulting in poor detection. Samples 004g02, 004g04 and 004g05 had five to eight mismatches compared to the probe, with one of them residing at the ligation site resulting in no probe binding. Moreover, sample 004g04 also had an insertion of two adenine bases. Finally, the mutant sample 005g01 had one mismatch close to the ligation site and was not detected.

Similar sensitivities were reported for the mutations detected in the NRTI kit using the VQA samples. Of the nine samples evaluated for codon 65, all were wild-type as determined by genotyping. However, only three of these samples (004g03, 004g04 and 005g01) were correctly identified by OLA (Table 1). Sample 004g04 had two mismatches on the common probe six bases from the ligation site, samples 004g03 and 005g01 had four and five mismatches, respectively, but these were spaced evenly throughout the probe. Samples 004g02 and 004g05 were both not detected and had six mismatches compared to the probes (Fig. 1c). The remaining four samples had mismatches close to the ligation site as well as other mismatches, which would have resulted in no or poor probe binding.

Only seven of the VQA samples were used to evaluate codon 151, of which all were wild-type as determined by genotyping. Six of these samples (004g02, 004g03 and 004g 05, 005g 02, 005g 03 and 005g 01) were correctly identified by OLA (Table 1). Sample 005g02 had 100% homology with the probes; samples 004g02, 004g03 and 004g05 had one mismatch far from the ligation site. The remaining two samples (005g03 and 005g05) had two mismatches (Fig. 1d). The seventh wild-type sequence (004g04) had a mismatch at the site of ligation compared to the probe.

Analysis of the sequences for codon 184 revealed that of the seven samples, five were wild-type and two were mutant as determined by genotyping. However, only two of these samples (005g03 and 005g05) were correctly identified by OLA (Table 1). The three wild-type sequences (004g02, 004g04 and 004g05) had a mismatch two bases from the ligation site and had five mismatches compared to the probe. The remaining two mutant sequences (004g03 and 005g02) both had a mismatch two bases from the ligation site as well as three mismatches compared to the probe, which would have contributed to poor probe binding (Fig. 1e).

The last codon analysed on seven VQA samples was codon 215, of the seven subtype samples, three were wild-type and

four were mutant for T215Y as determined by genotyping. Six samples (004g02, 004g03, 004g04, 004g05, 005g03 and 005g05) were correctly identified by OLA (Table 1), sequence analysis revealed they had between four and six mismatches compared to the probe, however, these mismatches were far from the ligation site. The sample not identified (005g02) had three mismatches, two of which were close to the ligation site (Fig. 1f).

3.2.2. Results for subtype C samples

Forty-four subtype C samples were selected from a cohort of 102 patients referred for routine genotypic resistance testing (in-house numbering: DR001-102). The subtype C samples were tested for the same codons as the subtype B samples described above. For codon 103, of the 20 subtype C samples, 9 were wild-type and 11 were mutant as determined by genotyping. However, only five of these samples were cor-

		Mismatches: Wild-type	Common
(a) **K103N**:			
wild-type:	ACA TCC CGC AGG GTT AAA AAA GAA **A*** AAA TCA GTA ACA GTA CTG GAT GTG GGT		
Samples correctly identified by OLA:			
005g03	ACA TCC CGC AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA CTR GAT GTG GGT	0	0
005g05	ACA TCC CGC AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA CTA GAT GTG GGT	0	0
Samples not correctly identified by OLA:			
004g01	ACA TCC TGC AGG GAT AAA AAR GAA C *AAA TCA GTA TCA GTA ATA GAT GTG GGT	2	2
004g02	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
004g03	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
004g04	TCA TCC CGC GGG ATT AAA AGA GAA A *AAA TCG GTA ACA GTA CTA GAT GTG GGG	3	2
004g05	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
005g01	ACA TCC CGC AGG GTT AAA AAA GAG C *AAA TCA GTA ACA GTA CTA GAC ATA GGT	1	3
005g02	ACA TCC TGC AGG GAT AAA AAR GAA C *AAA TCA GTA TCA GTA ATA GAT GTG GGT	2	2
(b) **Y181C**:			
wild-type:	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CAT GGA TGA TTT GTA TGT A		
Samples correctly identified by OLA:			
004g03	ACA GAA TCC AGA AAT AGT TAT CTA *TCA ATA YGT GGA TGA TTT GTA TGT A	2	2
005g02	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CAT GGA TGA TTT GTA TGT A	0	0
005g03	AAA GAA TCC AGA CAT RGT TAT CTA *TCA ATA CGT GGA TGA TTT GTA TGT A	3	1
005g05	AAA GAA TCC AGA CAT RGT TAT CTA *TCA ATA CGT GGA TGA TTT GTA TGT A	3	1
Samples not correctly identified by OLA:			
004g01	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CHT RGA TGA TTT GTA TAT A	0	3
004g02	ACA GAA TCC AGA AAT ART TAT CTA *YCA ATA TGT GGA TGA TTT GTA TGT A	3	3
004g04	ACA AAA AATCC AGA GAT GGT GAT CTA*CCA ATA TAT GGA TGA TTT ATA TGT A	5	3
004g05	ACA GAA TCC AGA AAT ART TAT CTA*YCA ATA TGT GGA TGA TTT GTA TGT A	3	3
005g01	ACA AAA TCC AGA CAT AGT TAT CTA *TCA ATA CGT GGA TGA TTT GTA TGT A	0	1
(c) **K65R**:			
wild-type:	CTC CAG TAT TTG CCA TAA AGA **A*** RAA AGA CRG TAC TAA ATG GAG AA		
Samples correctly identified by OLA:			
005g02	CTC CAG TAT TTG CTA TAA AAA A *GAA AAA CAG TAC TAA ATG GAG AA	2	1
005g03	CTC CAG TAT TTG CCA TAA AGA A *AAA GGA CAG TAC TAA ATG GAG AA	0	1
005g05	CTC CAG TAT TTG CCA TAA AGA A *AAA GGA CAG TAC TAA ATG GAG AA	0	1
Samples not correctly identified by OLA:			
004g01	CTC CAA TAT TTG CNA TAA AGA A *GAA AAA CAG TAC TAA ATG GAG AA	1	1
004g02	CTC CAA TAT TTG CTA TAA AGA A *GAA AAA CAG TGA TCA ATG GAG AA	2	4
004g03	CTC CAA TAT TTG CNA TAA AGA A *GAA AAA CAG TGA TAA ATG GAG AA	1	3
004g04	CTC CAG TAT TTG CCA TAA AGA A *AAA AGA TAG TAC CAA ATG GAG AA	0	2
004g05	CTC CAA TAT TTG CTA TAA AGA A *GAA AAA CAG TGA TCA ATG GAG AA	2	4
005g01	CTC CAA TAT TTG CTA TAA AAA A *GAA AAA CAG TAC TAA ATG GAG AA	3	2

Fig. 1. VQA proficiency panel samples used in OLA detection assay: (a) K103N, (b) Y181C, (c) K65R, (d) Q151M, (e) M184V and (f) T215F/Y. (1) The top line shows the wild-type and common probe sequences (underlined). (2) The bold base indicates the site of mutation and the asterisk indicates the ligation site. (3) Below the probes are the aligned VQA sequences, the shaded bases reflect differences from the probes. (4) The sequences are separated into those that were correctly identified from those that were missed. (5) The number of mismatches on the far right indicates the amount of mismatches relative to wild-type and common probes, respectively. (6) The numbers shaded are mismatches that occur near the ligation site.

Fig. 1. (*Continued*).

rectly identified by OLA (Table 1), of which four sequences were obtained (DR010, DR017, DR037 and DR094). Samples DR010, DR017 and DR037 had wild-type sequences that showed two to three mismatches to the probe (Fig. 2a). The fourth correctly identified sequence (DR094) had a mutant sequence that revealed three mismatches compared to the probe. The five undetected wild-type sequences (DR016, DR019 and DR044) had seven, six and four mismatches compared to the probe, whereas DR084 had a mismatch one base from the ligation site and DR029 had a mismatch at the ligation site both had four mismatches compared to the probe (Fig. 2a). Of the four sequences obtained for undetected mutants, (DR028, DR047, DR050, DR095) it was found that mismatches four to five compared to the probe and these mismatches occurred close to the ligation site.

For codon 181, of the 17 subtype C samples, 14 were wild-type and 3 were mutant as determined by genotyping. However, only four of these samples were correctly identified by OLA (Table 1) of which two sequences were obtained (DR029 and DR068). Samples DR029 and DR068 had wild-type sequences that showed four and six mismatches to the probe, respectively (Fig. 2b). One sample (DR012) was incorrectly detected as a false positive, upon sequence analysis it was found that this sample had mismatches on both sides of the ligation site, resulting in sub-optimal ligation. 11/17 samples were not detected, of the 10 sequences obtained (DR006, DR084, DR048, DR041, DR095, DR094, DR044, DR037 and DR028), analysis revealed that mismatches ranged from 6 to 29 compared to the probe. Moreover, a mutant sample (DR021) had four mismatches compared to the probe close to the ligation site (Fig. 2b).

Nineteen subtype C samples, 15 were wild-type and 4 mutants for codon 65. The 0/19 samples were detected correctly (Table 1). Sequence analysis revealed that no probe binding was a result of a consistent base change two bases away from the ligation site on the wild-type/mutant probes of samples

DR010, DR016, DR019, DR028, DR029, DR037, DR050, DR084, DR006, DR092, DR095, DR098, DR044, DR068, DR069 and DR047. Sample 17 had 10 mismatches compared to the probe. Moreover, all samples with a mismatch close to the ligation site also had mismatches ranging from 4 to 31 (Fig. 2c).

For codon 151, of the 24 subtype C samples, 23 were wild-type and 1 mutant as determined by genotyping. However, only 18 samples (DR006, DR012, DR016, DR017, DR019, DR030, DR032, DR037, DR044, DR055, DR060, DR061, DR064, DR069, DR082, DR092 and DR095) were correctly identified by OLA (Table 1). The 5/24 samples (DR005, DR028, DR041, DR091 and DR094) had a base change within five bases from the ligation site on the wild-type/mutant probe (Fig. 2d).

For codon 184, of the 24 subtype C samples, 15 were wild-type and 9 mutants as determined by genotyping. However, only five samples (DR006, DR017, DR041, DR055 and DR088) were correctly identified by OLA (Table 1). 19/24 Samples (DR005, DR012, DR016, DR019, DR028, DR030, DR032, DR037, DR044, DR060, DR061, DR064, DR068, DR069, DR082, DR091 DR092, DR094 and DR095) had a base change within five bases from the ligation site on the wild-type/mutant probe (Fig. 2e).

For codon 215, of the 24 subtype C samples, 11 were wild-type and 13 mutants as determined by genotyping. However,

(a) **K103N:**		Mismatches: Wild-type	Common
wild-type:	ACA TCC CGC AGG GTT AAA AAA GAA **A** *AAA TCA GTA ACA GTA CTG GAT GTG GGT		
Samples correctly identified by OLA:			
DR010	ACA CCC AGG AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA CTG GAT GTG GGG	3	0
DR017	ACA TCC CGC AGG GTT AAA AAA GAA A *AAA TCA GTA ACA GTA ATA GAT GTG GGG	0	2
DR037	ACA CCC AGC AGG GTT AAA AAA GAA A *AAA TCA GTG ACA GTA CTG GAT GTG GGG	2	1
DR094	ACA CCC AGC AGG GTT AAA AAA GAA C *AAA TCA GTG ACA GTA CTG GAT GTG GGG	2	1
Samples not correctly identified by OLA:			
DR016	ACA CCC AGG AGG GTT AAA AAA GAA A *AAA TCA GTG ACA GTA CTA GAT ATA GGG	3	4
DR019	ACA CCC AGC AGG GTT ACA GAA GAA A *AAA TCA ATG ACA GTA CTG GAT GTG GGG	4	2
DR028	ACA TCC AGG AGG ATT AAA AAA GAA C *AAA TCA GTG ACA GTA CTA GAT GTG GGG	3	2
DR029	ACA CCC AGC AGG GTT AAA AAA GAA T *AAA TCA GTG ACA GTA CTG GAT GTG GGG	5	1
DR047	ACA CCC AGC AGG GTT AAA GAA GAA C *AAA TCA ATG ACA GTA CTG GAT GTG GGG	3	2
DR050	ACA CCC AGC AGG GTT AAA AAA GAA C *AAA TCA ATG ACA GTA CTG GAT GTG GGG	2	2
DR084	ACA CCC AGC AGG ATT AAA AAA GAG A *AAA TCA GTA ACA GTA CTG GAT GTG GGG	4	0
DR095	ACA TCC AGC AGG GTT AAA AAA GAA C *AAA TCA ATG ACA GTA CTG GAC GTG GGG	1	2
DR044	ACA CCC AGC AGG GTT AAA AAA GAA A *AAA TCA GTG ACA GTA CTA GAT GTG GGG	2	2
(b) **Y181C**:			
wild-type:	ACA AAA TCC AGA CAT AGT TAT CT**A** *TCA ATA CAT GGA TGA TTT GTA TGT A		
Samples correctly identified by OLA:			
DR029	ACA AAA TCC AGA AAT AGT CAT CTA *TCA ATA TAT GGA TGA CTT GTA TGT A	2	2
DR068	AAA AAA TCC AGA CMT AGT TAT CTA *TCA ATA TGT GGA TGA CTT RTA TGT A	2	4
Samples not correctly identified by OLA:			
DR006	ACG AAA CCA AGA AAT AGA GAT CTG *TCA ATA CAT GGA TGA CTT GTA TGT A	6	1
DR012	ACA AAA TCC AGA CAW AGT TAT CTG *TCA ATA TAT GGA TGA TTT GTA TGT A	1	1
DR021	ACA AAA TCC AGA AGT AGT TAT CTG *TCA ATA CGT GGA TGA TTT RTA TGT A	2	2
DR084	AAA AAA CCC AGA CAT AGA TAT CTA *TCA ATA TAT GGA TGA CTT GCT TGT A	3	4
DR048	GAA AAC AAA ATC CAG ACA TAG TCA *TTT RTC AAT ACA TGG ATG ATT TGT A	14	12
DR041	GGG CAC AAA ATC CAG AMA TAG TCA *TCT GTC AAT ACG TGG ATG ACT TGT A	16	13
DR095	AAA AAA TCC AGA AAT AGT CAT TTA *TCA ATA TAT GGA TGA CTT ATA TGT A	5	3
DR094	ACA AAA CCC AGA AAT AGT CAT CTA *TCA ATA TGT GGA TGA CTT GTA TGT A	3	3
DR044	AAA AAA TCC AGA ACT AGT CAT CTA *TCA ATA TAT GGA TGA TCT GTA TGT A	4	2
DR037	GAG CAA AAA ATC CAG ACA TAG TCA *TCT ATC AAT ATG TGG ATG ACT TGT A	15	12
DR028	ACA AAA TCC AGA GAT AGT CAT CTA *TCA ATA TGT GGA TGA CTT GTA TGT A	2	3

Fig. 2. Subtype C samples used in OLA detection assay: (a) K103N, (b) Y181C, (c) K65R, (d) Q151M, (e) M184V and (f) T215F/Y. (1) The top line shows the wild-type and common probe sequences (underlined). (2) The bold base indicates the site of mutation and the asterisk indicates the ligation site. (3) Below the probes are the aligned subtype C sequences, the shaded bases reflect differences from the probes. (4) The sequences are separated into those that were correctly identified from those that were missed. (5) The number of mismatches on the far right indicate the amount of mismatches relative to wild-type and common probes, respectively. (6) The numbers shaded are mismatches that occur near the ligation site.

(c) **K65R**

wild-type: CTC CAG TAT TTG CCA TAA AGA **A***RAA AGA CRG TAC TAA ATG GAG AA

Samples not correctly identified by OLA:

DR010	CAA TAT TTG CCA TAA AAA AGA A*ACA GTG ATA AGT GGA GAA AAT TA	12	19
DR016	CTC CAG TAT TTG CCA TAA AAA A*GAA GAA CAG TGA TAA GTG GAG AA	1	6
DR017	CTC CAA TAT TTG CTA TCA AGA A *AAA GAA CAG CGA TAG ATG GAG AA	3	7
DR019	CTC CAG TAT TTG CCA TAA AAA A*GAA AAA CTC TGA GAG TTC TAA GTG GAG AA	1	22
DR028	CTC CAG TAT TTG CTA TAA AAA A*GAA AGA CAG TAC TAA GTG GAG AA	2	2
DR029	CTC CAA TAT TCG CCA TAA AAA A*GAA GAA CAG CAC TAG GTG GAG AA	2	6
DR037	CCC CAR TAT TTG CCA TAA AAA A*GAA GAA CAG TAC TAC GTG GAG AA	3	5
DR050	CTC CAG TAT TTG CCA TAA AAA A*GAA GGA CAG TAC TAA GTG GAG AA	1	3
DR084	CTC CAA TAT TTG CCA TAA AAA A*GAA GGA CAG TAC TAA GTG GAG AA	2	5
DR006	CTC CAA TAT TTG CCA TAA AAA A*GAA AGA TGG MAA TAA GTG GAG AA	2	5
DR092	CTC CAA TAT TTG CCA TAA AAA A*GAA GAA CAG TGA TAG GTG GAG AA	2	7
DR095	CCC CGG TGT TTG CTA TAA AAA A*GAA GGA CAG TAC TAA GTG GAG AA	5	5
DR098	CTC CAA TAT TTG CCA TAA AAA A*GAA GAA CAG TAC TAR GTG GAG GA	2	6
DR044	CTC CAA TAT TTG CCA TAA AAA R*GAA GGA CAG TAC TAA GTG GAG AA	2	3
DR068	CTC CAG TAT TTG TCA TAA AAA R*GAA GGA CGG TAY TAA GTG GAG AA	2	4
DR069	CTC CAG TAT TTG CCA TAA AAA G*GAA GAA CAG TAC TAA GTG GAG AA	1	4
DR047	CTC CAG TAT TTG CTA TAA AAA R*GAA GRA CAG TAC TAA GTG GAG AA	2	4

(d) **Q151M**

wild-type: CAG TAC AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC

Samples correctly identified by OLA:

DR006	CAA TAC AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR012	CAG TAC AAT GTR CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR016	CAR TAT AAT GTG CTA CCA CA*GGG ATG GAA AGG ATC AC	3	0
DR017	CAG TAC AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	0	0
DR019	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR030	CAA TAC AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR032	CAA TAT AAT GTG CTA CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR037	CAG TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	0	0
DR044	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR055	CAA TAC AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR060	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR061	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR064	CAR TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR068	CAA TAT AAT GTG CTT CCA AT* GGG ATG GAA AGG ATC AC	2	0
DR069	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG GTC AC	1	1
DR082	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR092	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR095	CAA TAT AAT GTG CTT CCA CA*GGG ATG GAA AGG ATC AC	1	0

Samples not correctly identified by OLA:

DR005	CAA TAT AAT GTA CTT CCA CA* AGG ATG GAA AGG ATC AC	3	1
DR028	CAA TAT AAT GTG CTT CCA CA* AGG ATG GAA AGG ATC AC	2	1
DR041	CAA TAT AAT GTG CTA CCA CA* AGG ATG GAA AGG ATC AC	3	1
DR088	CAG TAC AAT GTG TTG CCA CA*GGG ATG GAA AGG ATC AC	1	0
DR091	CAG TAT AAT GTG CTG CCA CA* AGG ATG GAA AGG ATC AC	1	1
DR094	CAA TAT AAT GTT CTT CCA CA* GGG GTG GAA AGG ATC AC	3	1

Fig. 2. (*Continued*)

(e)**M184V**

wild-type: AGA CAT AGT TAT CTA TCA ATA CG*TGG ATG ATT TGT ATG TAG GAT C

Samples correctly identified by OLA:

DR017	AGA AAT AAT CAT ATA TCA ATA CA*TGG ATG ACT TGT ATG TAG GAT C	4	1
DR041	AGA MAT AGT CAT CTG TCA ATA CG* TGG ATG ACT TGT ATG TAG GAT C	3	1
DR055	AGA CAT AGT TAT CTA TCA ATA CG*TGG ATG ACT TGT ATG TAG GAT C	0	1
DR088	AGA AAT AGT TAT CTA TCA ATA CA*TGG ATG ACT TGT ATG TAG CAT C	1	2

Samples not correctly identified by OLA:

DR005	AGA ACT AGT CAT CTA TCA ATA CA*TGG ATG ACT TGT ATG TAG CAT C	3	2
DR006	AGA AAT AGA CAT CTG TCA ATA CA*TGG ATG ACT TGT ATG TAG GAT C	4	1
DR012	AGA CAW AGT TAT CTG TCA ATA TA*TGG ATG ATT TGT ATG TAG GAT C	3	0
DR016	AGA AAT AGT TAT CTA TCA ATA TG*TRG ATG ACT TAT ATG TAG GAT C	3	3
DR019	AGA AAT AGT CAT CTA TCA ATA TA*TGG ATG ACT TGT ATG TAG CAT C	3	1
DR028	AGA CAT AGT CAT CTA TCA ATA TG*TGG ATG CAT TGT ATG TAG CAT C	3	3
DR030	AGA CAT RGT TAT CTA TCA ATA TA*TGG ATG ACT TGT ATG TAG GAT C	2	1
DR032	AGA AAT AGT CAT CTA TCA ATA TA*TGG ATG ACT TAT ATG TAG CAT C	3	3
DR037	AGA CAT AGT CAT CTA TCA ATA TG*TGG ATG ACT TGT ATG TAG CAT C	3	2
DR044	AGA ACT AGT CAT CTA TCA ATA TA*TGG ATG ATC TGT ATG TAG GAT C	4	1
DR060	AGA CAT AGT CAT CTA TCA ATA TA*TGG ATG ACT TGT ATG TAG CAT C	2	2
DR061	AGA AAT AGT CAT CTA TCA ATA TA*TGG ATG ACT TGT ATG TAG GAT C	3	1
DR064	AGA AAT GGT CAT CTR TCA ATA TG*TGG ATG ACT TGT ATG TAG SAT C	3	2
DR068	AGA CMT AGT TAT CTA TCA ATA TG*TGG ATG ACT TRT ATG TAG GAT C	3	2
DR069	AGA AAT AGA CAT CTA TCA ATA CA*TGG ATG ACT TGT GTG TAG GAT C	3	2
DR082	AGA AAT AGT CAT CTA TCA ATA TA*TGG ATG ACT TCT ATG TAG GAT C	3	2
DR091	AGA CAT AGT TAT CTA TCA ATA YG*TGG ATG ATC TRT ATG TAG GAT C	1	2
DR092	AGA AAT AGT CAT CTA TCA ATA TA*TGG ATG ACT TAT RTG TAG SAT C	3	4
DR094	AGA AAT AGT CAT CTA TCA ATA TG*TGG ATG ACT TGT ATG TAG GAT C	3	1
DR095	AGA AAT AGT CAT TTA TCA ATA TA*TGG ATG ACT TAT ATG TAG GAT C	1	2

(f) **T215F/Y**

wild-type: CAA CAT CTG TTG AGG TGG GGA TTT AC*CAC ACC AGA CAA AAA ACA TCA GAA

Samples correctly identified by OLA:

DR005	GAA CAT TTA TTA CAG TGG GGA TTT AC*CAC ACC AGA CAA GAA ACA TCA GAA	6	1
DR012	CAA CAT CTG TTG AAA TGG GGA TTT AC*CAC ACC AGA CAA GAA GCA TCA GAA	2	2
DR019	GAA CAC CTC TGG AAG TGG GGA TTT TA*CAC ACC AGA CAA GAA ACA TCA GAA	5	1
DR028	GAC CAT CTA TTA AAG TGG GGA TTT TA*TAC ACC AGA CAA GAA ACA TCA GAA	5	3
DR030	GAA CAT TTA TTG AGA TGG GAA TTT TA*CAC ACC AGA CAA GAA ACA TCA GAA	4	1
DR037	GAA CAC CTG TTA AAG TGG GGA TTT AC*CAC ACC AGA CRA GAA ACA TCA GAA	4	2
DR041	GAG CAT CTR TGG AGG TGG GGA TTT TA*CAC ACC AGA CAA KAA ACA TCA GAA	4	1
DR044	GAA CAT TTA TTG AAA TGG GGA TTT AC*CAC GCC GGA CAA GAA ACA TCA GAA	5	3
DR060	GAA CAT CTA TGG AAA TGG GGA TTT TA*CAC ACC AGA CAA GAA ACA TCA GAA	5	1
DR061	GAC CAT TTA TTA AGG TGG GGA TTT WM*CAC ACC AGA CAA GAA ACA TCA GAA	7	1
DR069	GAC CAT CTA CTA AAG TGG GGA TTT AC*CAC ACC AGA CAA GAA ACA TCA GAA	6	1
DR094	GAA CAC CTA TTG AAA TGG GGA TTT AC*CAC ACC AGA CAA GAA ACA TCA GAA	5	1
DR095	GAA CAT TTA TTA AGG TGG GGA TTT TA*CAC ACC AGA CAA GAA ACA TCA GAA	4	1

Samples not correctly identified by OLA:

DR006	GAR CAT CTA TTG ARG TGG GGR TTT AC*CAC ACC AGA YAA GAA GYA TCA GAA	5	4
DR016	GAA TAT CTC TGG AAA TGG GGA TTT TT*CAC ACC AGA CAA GAA ACA CCA GAA	6	1
DR017	GCT CAT CTA TGG AAT TGG GGA TTT TT*TAC ACC AGA ACA AAA GCA TCA GAA	7	4
DR032	GAA CAT CTA TTA AAG TGG GGA TTT TA*CAC ACC AGA CSA GAA ACA TCA GAA	4	2
DR055	GCA CAT TTA TTR AGA TGG GGA TTT AC *CAC ACC AGA CAA GAA ACA TCA GAA	5	1
DR064	GAG CAT CTA TTA ARG TGG GGA TTT AC *YAC ACC AGA CAA GAA ACA YCA GAA	5	3
DR068	GAA CAT TTA TTG AGA TGG GGA GTT AC *CAC ACC AGA CAA GAA ACA TCA AAA	5	2
DR082	GAA CAT CTA TTA AGG TGG GGA TTT WM*CAC ACC AGA CAA GAA ACA TCA GAA	5	1
DR088	CAG CAT CTG TGG AAG TGG GGA TTT TA *CAC ACC AGA CAA GAA GCA TCA GAA	3	1
DR091	GAG CAT CTG TTG ARG TGG GGG CTT AC *YAC ACC AGA CCA GAA GCA TCA GAA	5	4
DR092	GAA CAT CTA TTG AAG TGG GGA TTC TG *TAC CCC AGA CCA GAA ACA TCA GAA	5	4

Fig. 2. (*Continued*).

only 13 samples (DR005, DR012, DR019, DR028, DR030, DR037, DR041, DR044, DR060, DR061, DR069, DR094 and DR095) were correctly identified by OLA (Table 1). 11/24 Samples (DR006, DR016, DR017, DR032, DR055, DR064, DR068, DR082, DR088, DR091 and DR092) had a base change within five bases from the ligation site on the wild-type/mutant probe (Fig. 2f).

4. Discussion

The OLA assay has been proposed as a simple and cost effective alternative to conventional genotypic sequencing assays for resource constrained environments such as South Africa (Beck et al., 2002). Limited evaluation of this assay has been conducted in non-B subtypes (Vega et al., 2003). This study was undertaken to evaluate OLA for the use of resistance testing in HIV-1 subtype C samples. The main classes of drugs proposed in the national ARV roll-out program in South Africa are combinations of NNRTIs and NRTIs. For this reason, we evaluated codons included in the NNRTI and NRTI kits supplied by the NIH reagent program and did not focus on the protease inhibitor mutations.

The OLA assay involves an RT-PCR and an ELISA-based detection step as described by Beck and Frenkel (2002). In order to reduce the turn-around-time and limit potential contamination in a routine laboratory setting, a one-step RT-PCR was introduced. This step was found to reduce the amplification time from about 7 to 5 h. In order to improve the efficiency of the PCR amplification in subtype C, primers from the kit were replaced with a pair designed specifically for the reverse transcriptase region of the *pol* gene of subtype C HIV-1. These subtype C specific primers were also able to amplify the subtype B plasmids supplied in the OLA kit thus eliminating the need for an extra set of primers.

Overall all codons investigated were poorly identified in both the HIV-1 subtype C and subtype B samples evaluated as compared to the mutations identified by genotyping (Table 1). Upon further analysis of the sequences of the subtype C samples obtained using ViroSeq™, it was found that several bases could be changed to potentially decrease the number of mismatches between the probes and the subtype C samples and thus improve ligation (Table 2).

In summary, the K103N proposed probe changes would reduce mismatches to less than four per sample and thus potentially improve ligation. It is uncertain how to address the issue of samples containing mismatches at the ligation site, for example the K103S polymorphism (VQA samples). This inability of the OLA assay to efficiently discriminate between major mutations and polymorphisms in the ligation area has also been demonstrated by Beck et al. (2002) for codon V82A where there was an additional base change at the site of ligation. The common probe for Y181C would require at least three changes, which should improve the binding of this probe, especially as one of the recommended changes is close to the ligation site. However, the mutant/wild-type probes have several mismatches (e.g., M184V, Y188H/C and G190A) when compared to subtype C sequences that were not consistent across samples studied and there appears to be little that could be changed to improve binding of these probes.

Several recommendations could also be made for the mutations associated with nucleoside reverse transcriptase inhibitors. For the K65R, four bases in the probes that when changed could facilitate improved binding. Two of these changes flank the ligation site and may thus significantly influence ligation. The Q151M probes are relatively well suited for subtype C, a mixed population of probes incorporating an A instead of a G in the common probe would allow for ligation to occur in order to obtain a result for identifying both the mutant and wild-type species. The M184V wild-type and mutant probe needs to be modified at two bases from the ligation site and perhaps a mixed population of probes would be ideal for detection. The T215F/Y probes were only able to tolerate four mismatches after which no binding occurred. There were no significant mismatches within the ligation site for the T215F/Y probes, however there were variable mis-

Table 2
Mutations associated with NNRTIs

K103N
ACA TCC CGC AGG GTT AAA AAA GAA A*AAA TCA GTA ACA GTA CTG GAT GTG GGT
ACA **C**CC **A**GC AGG GTT AAA AAA GAA A*AAA TCA GT**G** ACA GTA CTG GAT GTG GGT

Y181C
ACA AAA TCC AGA CAT AGT TAT CTA*TCA ATA CAT GGA TGA TTT GTA TGT A
ACA AAA TCC AGA CAT AGT TAT CTA*TCA ATA **T**AT GGA TGA **C**TT **R**TA TGT A

K65R
CTC CAG TAT TTG CCA TAA AGA A*RAA AGA CRG TAC TAA ATG GAG AA
CTC CA**R** TAT TTG CCA TAA A**A**A A*RAA **G**GA CRG TAC TAA **G**TG GAG AA

Q151M
CAG TAC AAT GTG CTT CCA AT*GGG ATG GAA AGG ATC AC
CA**R** TA**Y** AAT GTG CTT CCA AT*RGG ATG GAA AGG ATC AC

M184V
AGA CAT AGT TAT CTA TCA ATA CG*TGG ATG ATT TGT ATG TAG GAT C
AGA CAT AGT **Y**AT CTA TCA ATA **Y**G*TGG ATG A**Y**T T**R**T ATG TAG GAT C

T215F/Y
CAA CAT CTG TTG AGG TGG GGA TTT AC*CAC ACC AGA CAA AAA ACA TCA GAA
CAA CAT CTG TT**R** A**RR** TGG GG**R** TTT AC*CAC ACC AGA CAA **R**AA **R**CA TCA GAA

(1) The top sequence is the present probe's design. (2) The asterisks represent the ligation site. (3) The lower sequence is that of the proposed subtype C specific probe. (4) The wild-type probe in underlined, the common one probes follows. The bases in bold and underlined are those that would be changed to help in subtype C sample detection.

matches within each probe that contributed to the samples not being detected.

The main reason for inefficient probe binding in all the codons examined was the presence of several polymorphisms located in the patients' sequence, resulting in decreased complementarity to the probes. Beck et al. (2002) and Edelstein et al. (1998) both reported that their indeterminate probe binding was attributed to mismatches three bases from the ligation site or two or more base changes in the patient specimen in the region complimentary to either of the ligation probes. These proposed changes need to be evaluated to ascertain whether detection would be improved in the subtype C environment. The probes used to identify the resistance mutations in OLA are in the region of 40–50 bases long and due to the error-prone nature of HIV replication, the chances of mismatches existing in the probe regions is high.

The initial set up of the OLA assay was expensive since routine laboratories do not carry the chemicals required in the many buffers for this assay. However, the cost of a single sample (assuming a full plate of 43 samples is run), including PCR, would cost approximately US$ 20 per six mutations analyzed. This excludes the labour component, which is significant since the assay may need to be performed over several days.

In conclusion, the modifications made to the OLA PCR step improves both cost and reduces turnaround time making OLA more suitable for a routine setting. However, the OLA in its current format is unable to effectively differentiate between major mutations conferring drug resistance or subtype related polymorphisms, which may confer resistance in the ligation area. Further work is needed to establish whether probe changes suggested in this paper improve the detection of subtype C resistance mutations.

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