INNOVATIVE AND AFFORDABLE HIV-1 DRUG RESISTANCE TESTING FOR RESOURCE LIMITED SETTINGS

Submitted in fulfilment of the requirements for the degree of Masters Medical Science at the Department of Virology, Nelson R Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

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By

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PREFACE

The study described in this thesis was carried out in the Department of Virology, National Health Laboratory Services (NHLS), in the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, in Durban, South Africa. It was conducted between February 2021 and January 2022 under the supervision of Dr Benjamin Chimukangara.

AUTHOR'S DECLARATION

I, Mr Sontaga Cris Manyana, declare that the entirety of the work contained therein is my own original work, and that I am the author of this work. The work in this thesis has not been used in any form by any person or submitted to any tertiary institution for the purposes of obtaining a degree or any other qualification. Some of the work has already been published in peer-review journals in-line with the thesis guidelines of University of KwaZulu-Natal. I have taken care in all respects to honour the intellectual property rights of others and have acknowledged the contribution of others work where it was used in the text, figures and or tables.

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PUBLICATIONS

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Publication 2

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Publication 3

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Other related publications during the period of study

Publication 4

Chimukangara B, Lessells RJ, Sartorius B, Gounder L, **Manyana S**, Pillay M, et al. HIV-1 drug resistance in adults and adolescents on protease inhibitor-based antiretroviral treatment in KwaZulu-Natal Province, South Africa. J Glob Antimicrob Resist. 2021; S2213-7165(21)00249-6.

Publication 5

Guarido MM, Motlou T, Riddin M, MacIntyre C, **Manyana SC**, Johnson T, Schrama M, Gorsich E, Brooke B, Almeida A.P, et al. Potential Mosquito Vectors for Shuni Virus, South Africa, 2014–2018. *Emerg. Infect. Dis. J.* 2021, *27*, 3142.

TABLE OF CONTENTS

PREFACE ii
AUTHOR'S DECLARATION iii
ACKNOWLEDGEMENTS iv
PUBLICATIONS v
TABLE OF CONTENTS vi
LIST OF FIGURES viii
LIST OF TABLES ix
APPENDICES x
LIST OF ABBREVIATIONS xi
ABSTRACT xiv
CHAPTER 1: INTRODUCTION 1
1.1 Background 1
1.2 Literature Review 4
1.2.1 Human Immunodeficiency Virus (HIV) 4
1.2.2 HIV life cycle 5
1.2.3 Antiretroviral drugs, mechanisms of action, and drug resistance 6
1.2.4 Antiretroviral therapy in South Africa 12
1.2.5 HIV drug resistance (HIVDR) 13
1.2.6 Point mutation assays 16
1.2.7 Allele-specific polymerase chain reaction (AS-PCR) 16
1.2.8 Pan-degenerate amplification and adaptation (PANDAA) 16
1.2.9 Oligonucleotide ligation assay (OLA) 17
1.2.10 Sanger sequencing and next generation sequencing (NGS) 17
1.3 Study Rationale and Justification 17
1.4 Study aim 18
1.5 Study objectives 18
1.6 Study method and settings 18
1.6.1 Inclusion and exclusion criteria 18
1.6.2 CAPRISA and the Advanced Clinical Care Programme 19
1.6.3 National Health Laboratory Service (NHLS) 19
1.6.4 Sample collection and transport 20
1.6.5 Ethical approval 20
1.7 Thesis outline 20

CHAPTER 2: HIV-1 DRUG RESISTANCE GENOTYPING IN RESOURCE LIMITED SETTINGS: CURRENT AND FUTURE PERSPECTIVE IN SEQUENCING TECHNOLOGIES 22

CHAPTER 3: AFFORDABLE DRUG RESISTANCE GENOTYPING OF HIV-1 REVERSE TRANSCRIPTASE, PROTEASE AND INTEGRASE, FOR RESOURCE LIMITED SETTINGS 33

CHAPTER 4: AFFORDABLE METHOD FOR GENOTYPING HIV-1 REVERSE TRANSCRIPTASE, PROTEASE AND INTEGRASE GENES: AN IN-HOUSE PROTOCOLS 70

CHAPTER 5: SYNTHESIS 91

5.1 Discussion 91

5.2 Recommendations for future research and policy 95

5.4 Conclusion........ 96

REFERENCES 97

APPENDICES 109

LIST OF FIGURES

Figure 1 The structure of HIV-1 ... 4

Figure 2 HIV-1 life cycle... 6

Figure 3 Mechanisms of action of nucleoside reverse transcriptase inhibitors ... 8

Figure 4 Mechanisms of action of non-nucleoside reverse transcriptase inhibitors ... 9

Figure 5 Mechanisms of action of protease inhibitors ... 10

Figure 6 Mechanisms of action of integrase inhibitors ... 11

Figure 7 HIV drug resistance mutation nomenclature ... 13

Figure 8 Phenotypic testing inhibitory concentration curve... 14

Figure 9 Summary of wet laboratory steps in genotypic HIVDR testing ... 15

Figure 10 Comparison of discordant mutation positions between IDR and CAP103 sequences ... 93

LIST OF TABLES

Table 1. Progress towards UNAIDS 95-95-95 goals by province, in South Africa 2

Table 2. Previous and current first- and second-line regimens for adults living with HIV in South Africa 12

Table 3. Comparative cost analysis for in-house HIVDR methods in RLS 94

APPENDICES

Appendix 1 Biomedical research ethics approval 109

Appendix 2 CAP 103 approval letter of support to use remnant samples 110

Appendix 3 CAP 103 study ethics approval from Biomedical Research Ethics Committee 111

Appendix 4 Supplementary material to manuscript entitled “Affordable drug resistance genotyping of HIV-1 reverse transcriptase, protease and integrase genes, for resource limited settings” 112

LIST OF ABBREVIATIONS

3TC	Lamivudine
ABC	Abacavir
ADR	Acquired drug resistance
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral treatment
ARVs	Antiretrovirals
ATVr	Ritonavir-boosted atazanavir
AS-PCR	Allele-specific polymerase chain reaction
AZT	Zidovudine
cDNA	Complementary deoxynucleic acid
CRFs	Circulating recombinant forms
d4T	Stavudine
DBS	Dried blood spot
ddI	Didanosine
DLV	Delavirdine
dNTP	Deoxynucleotide triphosphates
DTG	Dolutegravir
DoH	Department of Health
DOR	Doravirine
DOV	Department of Virology
DRVr	Ritonavir-boosted darunavir
EFV	Efavirenz
ETR	Etravirine

EVG	Elvitegravir
FDA	Food and Drug Administration
FTC	Emtricitabine
GTR	Genotypic drug resistance testing
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HIVDR	Human immunodeficiency virus drug resistance
IALCH	Inkosi Albert Luthuli Central Hospital
IN	Integrase inhibitors
INSTI	Integrase strand transfer inhibitor
KZN	KwaZulu-Natal province
LPVr	Ritonavir-boosted lopinavir
mRNA	Messenger ribonucleic acid
NADIA	Nucleosides and Darunavir/Dolutegravir in Africa
NGS	Next generation sequencing
NHLS	National Health Laboratory Service
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
OH	Hydroxyl group
OLA	Oligonucleotide ligation assay
PANDAA	Pan-degenerate amplification and adaptation
PEPFAR	United States President’s Emergency Plan for AIDS Relief
PDR	Pretreatment drug resistance
PCR	Polymerase chain reaction

PIs	Protease inhibitors
PMA	Point mutation assay
PR	Protease
qPCR	Quantitative polymerase chain reaction
RAL	Raltegravir
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
RLS	Resource limited settings
SATURN	Southern African Treatment and Resistance Network
SSA	Sub-Saharan Africa
TAMs	Thymidine analogue mutations
TLD	Tenofovir lamivudine and dolutegravir
TDF	Tenofovir
TDR	Transmitted drug resistance
UNAIDs	Joint United Nations Programme on HIV/AIDS
URF	Unique recombinant form
UTT	Universal test and treat
VL	Viral load
VF	Virological failure
WHO	World Health Organization

ABSTRACT

Background: HIV drug resistance (HIVDR) remains a major threat to achieving sustainable viral suppression on antiretroviral treatment (ART). Most countries including those in resource limited settings (RLS) have adopted use of dolutegravir (DTG), a more potent integrase strand transfer inhibitor, leading to an increase in the demand for integrase resistance testing. Current HIVDR testing methods in RLS focus on genotyping the HIV protease (PR) and reverse transcriptase (RT) genes, separate from the integrase (IN) gene. However, amplification of PR and RT separate from IN is expensive and increases the workload for HIVDR genotyping. Therefore, affordable and labour efficient methods that genotype all relevant HIV-1 genes (i.e., the PR, RT and IN genes) are required to guide clinical decisions, especially in RLS where cost is a major limiting factor. Thus, this study aimed to design an affordable in-house HIVDR genotyping method suitable for use in RLS.

Methods: Remnant plasma samples were obtained from a CAPRISA 103 study and viral RNA was extracted from 500µl of plasma. We validated the assay using remnant plasma samples from an external quality assessment (EQA) programme. Complimentary DNA synthesis and first-round PCR were performed followed by second-round nested PCR which was designed to amplify an ~2.9kb HIV-1 *pol* region (PR, RT and IN genes) using 1% gel electrophoresis. Successful second-round nested PCR products were purified using ExoSAP-IT Express PCR Product Cleanup reagent. Sanger sequencing was performed and quality of the sequences were manually edited using Geneious Prime software. HIVDR mutations were assessed using the Stanford HIV drug resistance database. HIVDR mutations using the designed method were compared to previous results obtained on the same samples. Sequence quality was also evaluated using phylogenetic analysis in Geneious software with maximum likelihood tree reconstruction using a generalized time reversible model with proportion of invariable sites and gamma distribution (GTR + I + G), and with 100 bootstrap replicates. Method cost-estimates were done by comparing costs and turn-around time to current genotyping methods.

Results: Of 115 plasma samples obtained, 19 samples were not processed due to inadequate plasma volume. Of the 96 processed, we obtained sequence data for 78 (81%). Of those, 75 (96%) had at least one HIVDR mutation in the PR and RT

genes, with no major-IN mutations observed. Only one sample had an E157Q INSTI-accessory mutation. When compared to previous genotypes, only 2/79 (3%) had different phenotypic predictions that affected the choice of subsequent regimens. Of 7 EQA samples, 4 were HIV-1C, 2 were HIV-1D, and 1 was HIV-1A. Genotypic resistance data generated using the IDR method showed 100% concordance with EQA panel results. The overall cost per sample was estimated at ~US$43, with a turn-around time of ~15 hours.

Conclusion: We successfully designed an in-house HIVDR method suitable for genotyping HIV-1C PR, RT and IN genes, at an affordable cost of US$64 and shorter turn-around time reduced from ~21 hours to ~15 hours, compared to currently available methods. This HIVDR genotyping method accommodates changes in ART regimens and will help to guide HIV-1 treatment decisions in RLS.

CHAPTER 1: INTRODUCTION

1.1 Background

Since the beginning of the human immunodeficiency virus (HIV) epidemic, roughly 79.3 million (uncertainty bounds; 55.9 – 110 million) people have been infected by the virus, and 36.3 million (27.2 – 47.8 million) people have died of HIV-related illnesses [1]. By 2020, there were approximately 37.7 million (30.2 – 45.1 million) people living with HIV, with adults aged 15 years and above accounting for ~98% (36.0 million, 28.9 – 43.2 million) of all HIV infections [1]. The number of annual new HIV infections (all ages) decreased from 1.7 million [1.6 million – 2.3 million] to 1.5 million [1.0 million–2.0 million] between 2018 – 2020 [1]. There has also been modest global progress towards reaching the UNAIDS 95-95-95 goals that target eliminating HIV by 2030, with an estimated 84% (67–98%) of people living with HIV knowing their status, 73% (56–88%) of those receiving antiretroviral treatment (ART), and 66% (53–79%) of those being virally suppressed, by 2020 [1].

Despite the global reduction in number of people infected with HIV and continual progress towards reducing HIV infections, sub-Saharan Africa (SSA) remains the region largely affected, accounting for ~39% of all new HIV infections in 2020 [1,2]. East and Southern Africa are the most affected areas, with an estimated 20.6 million (16.8 million – 24.4 million) people living with HIV and accounting for 670 000 (470 000 – 930 000) of all new HIV infections reported from SSA in 2020 [1]. South Africa remains the epicentre of the HIV epidemic, being home to 1 in every 26 people living with HIV in SSA by 2020 [3]. In 2021, approximately 8.2 million people were living with HIV, with 200 000 new infections and 79 420 HIV-related deaths in South Africa alone [4]. With approximately 5.5 million people receiving ART by the end of 2020, the country has the most extensive HIV treatment programme globally, accounting for 1 in every 5 people receiving ART globally [3].

In South Africa, HIV prevalence varies markedly between provinces. For example, KwaZulu-Natal (KZN) province has the highest HIV prevalence in the country (i.e., >50% prevalence between the ages 15 to 25 years) [5], being home to over 1.6 million people living with HIV, and having approximately 42 000 new HIV infections in 2021 alone [6]. Nevertheless, the province has made significant progress towards achieving the UNAIDS 95-95-95 targets, having the highest proportion of HIV infected individuals knowing their status, and the highest proportion of individuals achieving virological suppression on ART. Table 1 shows the 2020 South African HIV epidemic provincial statistics provided

by the Thembisa model which is designed to answer policy questions relating to HIV prevention and treatment [7].

Table 1. Progress towards UNAIDS 95-95-95 goals by province in South Africa

Province	% of HIV positive individuals diagnosed	% of diagnosed individuals on ART	% of individuals on ART with virological suppression (VL <1000 copies/mL)
KwaZulu-Natal	92.5%	72.4%	91.7%
Limpopo	91.7%	73.3%	85.2%
Northern Cape	90.9%	83.0%	84.4%
Mpumalanga	90.6%	73.5%	88.1%
Eastern Cape	90.1%	61.6%	87.2%
Free State	89.2%	73.4%	91.5%
North West	89.2%	57.7%	87.9%
Gauteng	88.9%	60.9%	87.3%
Western Cape	88.7%	65.5%	89.8%
Overall	**90.2%**	**69.0%**	**88.1%**

ART, antiretroviral treatment; HIV, human immunodeficiency virus; RNA, ribonucleic acid; mL, millilitre; VL, viral load

The introduction of ART has reduced global mortality rates and prolonged the lifespan of people living with HIV. In addition, ART access has a population-level advantage in suppressing HIV replication and the inherent prevention of onward transmission of the virus, termed “treatment as prevention” [8]. With more than 20 antiretroviral (ARV) drugs approved by the Food and Drug Administration (FDA), the quality of life of most HIV-infected patients has significantly improved [9], and HIV has become a manageable chronic infection [10]. Over the past 10 years, there has been an impressive scale-up of ART roll-out in resource limited settings (RLS) [11]. Most countries in RLS have adopted the universal test-and-treat (UTT) approach which aims to reduce HIV infection through expanding prevention and treatment services [12]. This approach is now supported by various multinational groups, such as the United States President’s Emergency Plan for AIDS Relief (PEPFAR) [13]. In South Africa, the Department of Health (DoH) supplies ARVs through a public sector roll-out programme, which has experienced a substantial expansion in recent years, keeping with the World Health Organization’s (WHO) changes to ART guidelines. In partnership with PEPFAR, the South African DoH has focused on 27 high-burden districts throughout the country, linking people living with HIV to lifesaving ART to improve treatment adherence [14]. Regardless of the positive impact of UTT and PEPFAR activities

in the South African HIV treatment programme, the country's greatest challenge remains that of effectively monitoring viral loads of people on ART, with critical gaps in the viral load testing cascade resulting in long delays in switching people with virological failure. As such, there remains a problem with emergence and transmission of drug resistant virus, leading to poor virological outcomes [15].

Generally, there have been concerns over increasing levels of HIV drug resistance (HIVDR) in RLS among people initiating or re-initiating ART, with or without prior ART exposure (i.e., pretreatment drug resistance (PDR)) [11,16], and among people on life-long ART with acquired drug resistance (ADR). Treatment failure identification and switching individuals with poor virological outcomes is often slow in RLS [22]. In 2021 the World Health Organization (WHO) issued new guidelines recommending use of dolutegravir (DTG) an integrase strand transfer inhibitor (INSTI), with two nucleoside reverse-transcriptase inhibitors (NRTIs), namely tenofovir (TDF) and lamivudine (3TC) for first-line ART [18]. Results from the Nucleosides And Darunavir/Dolutegravir In Africa (NADIA) trial have supported this approach, showing adequate viral suppression (i.e., VL <400 copies/mL) at 96-weeks among individuals on DTG-based ART, including in those where NRTIs were predicted to have no activity, with the ARTIST study showing similar outcomes in viral suppression (i.e., VL <50 copies/mL) at 24-weeks follow-up [19,20]. However, the ADVANCE study trial showed inadequate viral suppression rates (i.e. VL <50 copies/mL) among individuals receiving DTG-based ART for ≥48-weeks, raising concerns over long-term viral suppression in a setting with high NNRTI-PDR [21]. Regardless of DTG effectiveness, emergence of DTG-associated resistance mutations has been reported and HIVDR remains a cause of concern [22].

HIVDR testing can be done as phenotypic or genotypic testing. Phenotypic drug resistance testing is done via *in vitro* susceptibility cultures where a patient's viral sample is grown with a series of drug concentrations [23]. Genotypic testing of HIVDR can be done through a method of dideoxy chain termination known as Sanger sequencing which is cheaper compared to phenotypic testing. Sanger sequencing also known as population sequencing, has been available since the 1970s and is the most common method used for HIVDR testing [24]. It reliably detects viral variants that are well represented within a viral pool (i.e., at >20%) [25]. In contrast, advanced genomic sequencing known as next-generation sequencing (NGS) has the ability to detect infrequent viral variants (i.e., at <20%) [26]. Also, various low-cost point mutation assays (PMA) have been developed to detect only specific viral mutations [27].

In genotypic testing, most affordable methods used in RLS have been based on in-house assays that amplify and detect HIVDR in the protease (PR) and reverse transcriptase (RT) genes, such as the Southern African Treatment and Resistance Network (SATuRN) drug resistance protocol [28]. This has largely been due to PIs, NRTIs and NNRTIs being the most common drugs used for HIV treatment. However, as more people receive DTG (INSTI-based ART), simple and affordable methods of detecting HIVDR in the integrase (IN) gene are becoming increasingly important. Therefore, we designed a single-assay method for detecting HIVDR mutations in all relevant HIV-1 genes (i.e., PR, RT, and IN) at an affordable cost, to help inform clinical decisions.

1.2 Literature Review

1.2.1 Human Immunodeficiency Virus (HIV)

HIV is a single-stranded ribonucleic acid (RNA) virus in the genus lentivirus within the family Retroviridae, and subfamily Orthoretrovirinae [29]. The HIV genome consists of nine genes that encode fifteen viral proteins [30]. Three major HIV genes; gag, pol, and env, code for structural proteins (matrix, capsid and nucleocapsid), enzymes (PR, RT, and IN) and envelope proteins (gp120 and gp41), respectively (Figure 1) [30]. The remaining six genes code for accessory proteins (vif, vpr, vpu/vpx, and nef), and regulatory proteins (tat and rev) [30].

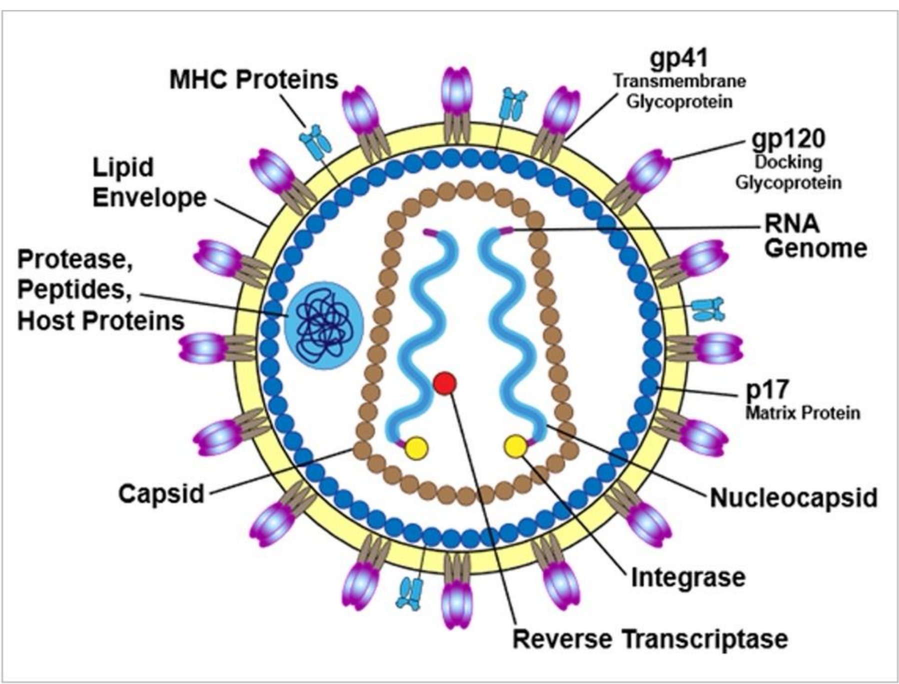

Figure 1 The structure of HIV-1

The figure shows structural proteins, enzymes and envelope proteins of the HIV genome.

(Reproduced as is from Tomé, C (2021, October 18). Human Immunodeficiency Virus Structure Retrieved October 2021, from https://mappingignorance.org/2013/01/10/towards-a-vaccine-against-hiv/fig1-4/)

HIV-1 is classified into four groups; major group (M), outlier group (O), non-major group (N) and the last identified group (P) [31]. Approximately ~99% of global HIV-1 infections are caused by group M viruses, which are divided into nine subtypes (A, B, C, D, F, G, H, J and K) as well as circulating recombinant forms (CRFs), and unique recombinant forms (URFs) [32]. Of these, HIV-1 subtype C (HIV-1C) accounts for almost 50% of all HIV-1 infections globally [33] and it is the dominant viral subtype in most RLS, including Asia and sub-Saharan Africa [34,35], with more than ~98% of infections in South Africa being attributed to HIV-1C [31].

1.2.2 HIV life cycle

HIV-1 entry into the host cell starts with HIV spike protein (gp120) binding to the primary receptor (CD4) and then to a co-receptor (i.e., either CCR5 or CXCR4) on the surface of the host cell (Figure 2) [36]. Once the CD4 membrane and HIV envelope fuse, gp41 inserts into the host cell membrane resulting in insertion of viral proteins into the CD4 cell [36]. Inside the cell, viral RNA is converted to double-stranded DNA by reverse transcriptase enzyme [37,38]. Following reverse transcription, viral DNA is transported through the nuclear pore to integrate into the host genome DNA in the nucleus [36]. Insertion of viral DNA into host DNA is mediated by viral enzyme integrase. The integrated form of viral DNA is known as proviral DNA [37]. The virus starts controlling transcriptionally active cells to produce viral messenger RNA (mRNA) using host enzyme RNA polymerase [39]. Translation of mRNA produces immature viral proteins which are cleaved into smaller functional proteins by HIV protease enzyme [38]. Viral proteins are assembled into new viral particles at the cell wall and bud off the host cell, complete maturation, and infect other cells [38]. HIV develops genetic variation during the replication cycles, largely due to its high replication rate and lack of proofreading activity during reverse transcription [40].

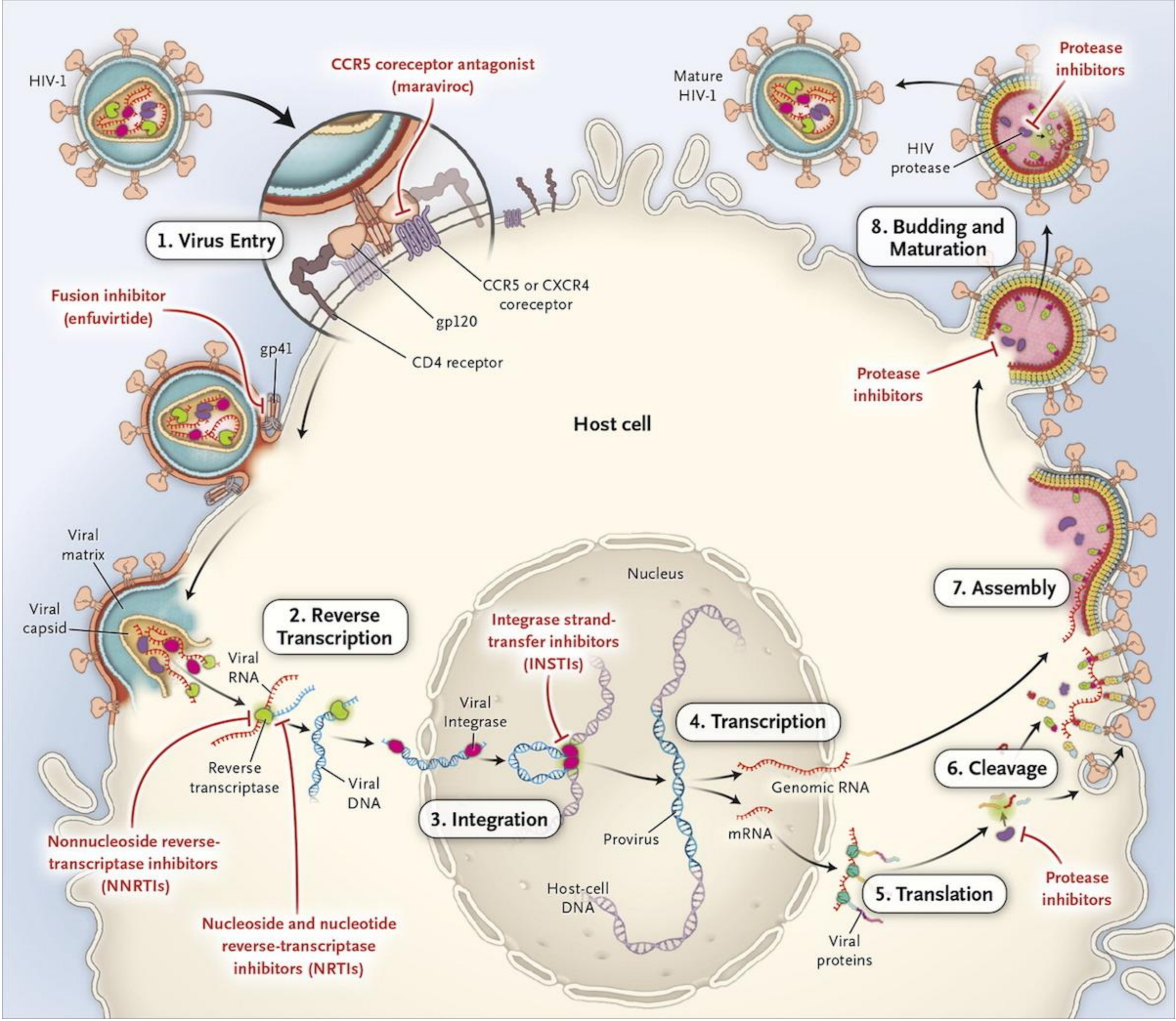

Figure 2 HIV-1 life cycle

This figure shows reproductive cycle of HIV-1. The figure also shows the different drug classes that can be used targeting different processes of the HIV-1 life cycle.

(Reproduced as is from Spach DH, Kinney RG (2021, October 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved October 2021, from https://www.hiv.uw.edu/go/antiretroviral-therapy/general-information/core-concept/all).

1.2.3 Antiretroviral drugs, mechanisms of action, and drug resistance

Antiretroviral drugs block HIV replication at different stages of the viral life cycle and are often administered as a combination of two or more drugs from at least two different drug classes [41]. The goal of ART is to suppress HIV to undetectable levels (i.e., VL <50 copies/mL), thereby reducing viral

transmission and emergence of HIVDR mutations [42,43]. Common ART drug classes in RLS include NRTIs, NNRTIs, PIs and INSTIs [43].

1.2.3.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

NRTIs have been a major component of first-line and subsequent ART regimens for decades. NRTIs are nucleoside analogues that compete with naturally occurring dNTPs to block viral reverse transcription [44]. Once incorporated, they terminate reverse transcription due to the lack of a 3'-hydroxyl group (3'-OH) [45]. Examples of commonly used NRTIs in RLS include, zidovudine (AZT), lamivudine (3TC), tenofovir disoproxil fumarate (TDF), and abacavir (ABC), with stavudine (d4T) having been phased-out due to adverse events such as peripheral neuropathy [46]. Despite NRTIs being relatively well tolerated, emergent NRTI-associated drug resistance mutations can lead to reduced antiviral activity.

There are two main mechanisms of NRTI drug resistance. In the first mechanism, resistance is mediated by mutations that allow reverse transcriptase enzyme to preferentially distinguish against NRTIs during DNA polymerization, blocking their addition to the DNA chain [47]. This mechanism of resistance is commonly observed when mutations occur near the nucleotide-binding site of RT. For example, M184V sterically inhibits productive binding of 3TC and emtricitabine (FTC) at the dNTP-binding site while, K65R, L74V, and Q151M slow down incorporation of NRTI-triphosphates [55]. The second mechanism involves mutations that promote hydrolytic unblocking of chain-terminating NRTIs through ATP excision activity, thereby allowing DNA synthesis to continue [47]. For example, thymidine analogue mutations (TAMs) such as M41L, D67N, K70R, L210W, T215YF, and K219QE, occur during treatment with thymidine analogues (i.e., AZT and d4T) and increase NRTI excision [47,49]. Consequently, mutations that distinguish against NRTIs are normally analogous with reduced enzymatic polymerase activity *in vitro*, whereas primer unblocking mutations are analogous with reduced enzymatic impairment [50]. Figure 3 shows the classical NRTI mechanisms of action and the subsequent effect of drug resistance.

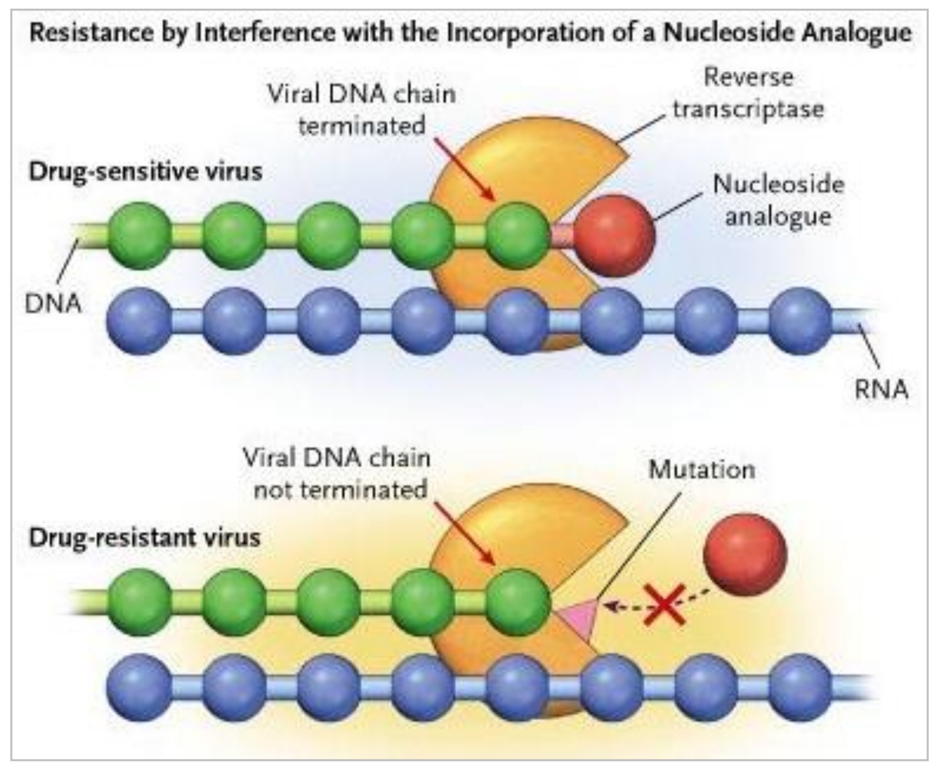

Figure 3 Mechanisms of action of nucleoside reverse transcriptase inhibitors
(Reproduced as is from François C, Allan JH (2021 October 22). HIV Drug Resistance. The New England Journal of Medicine. Retrieved October 2021, from https://www.nejm.org/doi/full/10.1056/NEJMra025195)

1.2.3.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs are non-competitive reverse transcriptase inhibitors that bind to a hydrophobic pocket of RT enzyme (Figure 4) [51]. This causes a change in the substrate-binding site reducing overall polymerase activity [52]. To date, six NNRTIs have been approved by the FDA. First-generation NNRTIs include nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV) [53], and second-generation NNRTIs include rilpivirine (RPV) and etravirine (ETR) [54,55]. The RT hydrophobic pocket is less conserved and development of a single mutation can result in drug resistance against an entire generation of NNRTIs (i.e., cross-resistance), hence they are known to have a low genetic barrier to resistance [52].

NNRTIs have largely been used as a backbone in first-line regimens, in RLS. When NNRTI resistance mutations emerge within the RT hydrophobic pocket, they reduce favourable interactions between the drug and RT-binding pocket [56,57]. The most common NNRTI resistance mutations that are observed in patients failing first-line NNRTI-based regimens include K103N, V106M, and Y181C [58]. Together

they cause intermediate to high-level resistance to all NNRTIs [59]. Figure 4 shows NNRTI mechanisms of action and the subsequent effect of drug resistance.

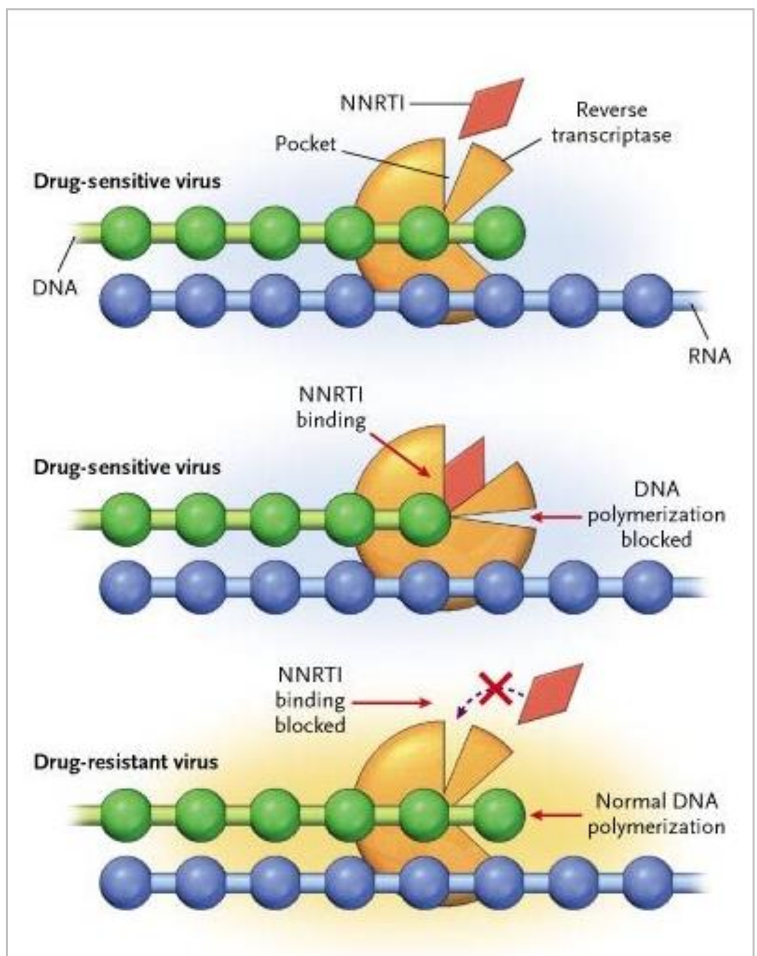

Figure 4 Mechanisms of action of non-nucleoside reverse transcriptase inhibitors (Reproduced as is from François C, Allan JH (2021 October 22). HIV Drug Resistance. The New England Journal of Medicine. Retrieved October 2021, from https://www.nejm.org/doi/full/10.1056/NEJMra025195)

1.2.3.3 Protease inhibitors (PIs)

PIs inhibit viral replication by competitively blocking the protease enzyme responsible for cleaving viral proteins to form functional virions [60]. In RLS, PIs have largely been reserved for use in second-line ART regimens in the adult population, replacing NNRTIs as the regimen backbone. Many active and non-active site PI mutations have developed overtime [61]. Resistance mutations in the substrate cleft decrease binding affinity between PI drugs and the mutant protease enzyme. Mutations elsewhere in the enzyme compensate for the reduced kinetics of enzymes with active site mutations or cause

resistance by changing enzyme catalysis, dimer stability, inhibitor binding kinetics, or changing the shape of the active site through long-range structural perturbations [61].

PIs are rapidly metabolized and cleared from the body by Cytochrome P450 (CYP450) 3A4 enzymes [62]. Inhibiting or inducing CYP450 may increase or reduce concentrations of ARV drugs, respectively [62]. Ritonavir has been shown to be a potent inhibitor of CYP3A4 enzyme. For this reason, ritonavir is administered together with other PI drugs to reduce the rate at which they are cleared, attaining therapeutic concentrations for desired periods, thus the term boosted-PI regimens [62,63]. However, PIs are generally not well tolerated and are associated with adverse events such as diarrhoea and nausea [63,64]. The most commonly used PIs in RLS include ritonavir-boosted atazanavir (ATVr) and ritonavir-boosted lopinavir (LPVr), with ritonavir-boosted darunavir (DRVr) used as a supplement drug in typical third-line ART [63]. Figure 5 shows PI mechanisms of action and the subsequent effect of drug resistance.

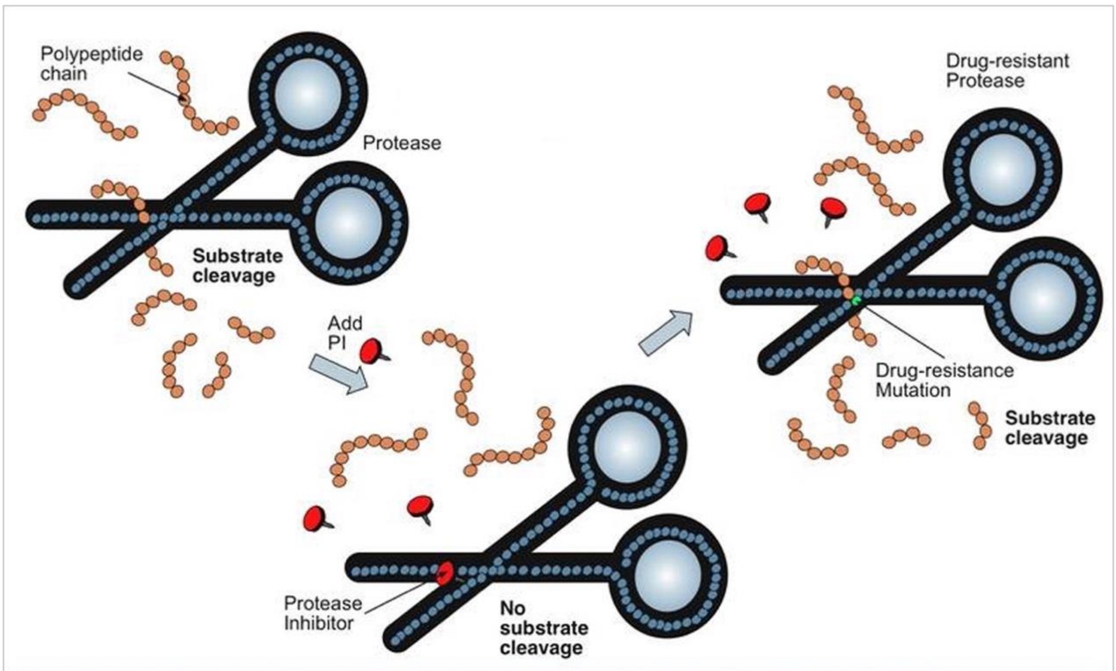

Figure 5 Mechanisms of action of protease inhibitors

(Reproduced with modifications from Immunopaedia. Advancing global immunology education. Immunopaedia.org. Retrieved October 2021, from https://www.immunopaedia.org.za/treatment-diagnostics/hiv-infection-treatment/arv-drug-information/)

1.2.3.4 Integrase strand transfer inhibitors (INSTIs)

INSTIs are a class of antiretroviral drugs that inhibit viral integration into host cell DNA (Figure 6) [65]. They are the only drug class that interacts with two essential components of the virus namely the integrase enzyme and viral DNA, at the point of integration [66]. INSTIs act by inhibiting HIV integrase from inserting viral DNA into the host cell's DNA [67]. Raltegravir (RAL) and elvitegravir (EVG) are first generation INSTIs. They have shown a low genetic barrier to resistance and cross-resistance, prompting development of second generation inhibitors [65]. DTG is a second generation INSTI that has been shown to have a higher genetic barrier to resistance, also showing activity against viral strains resistant to RAL and EVG [68]. DTG is the preferred drug in first-line ART and is administered with TDF and 3TC (i.e., NRTIs) as a single daily dose, in a regimen that is now commonly abbreviated as TLD [69]. Cabotegravir is also an FDA approved long-acting INSTI with a high genetic barrier to resistance. It can be administered as an oral tablet or as an injectable intramuscular long-acting suspension. This has the advantage of improving treatment adherence compared with administration of once-daily pill regimens [70,71]. Viral integrase can also develop resistant mutations leading to reduced viral susceptibility to INSTIs [72]. Figure 6 shows the INSTI mechanisms of action.

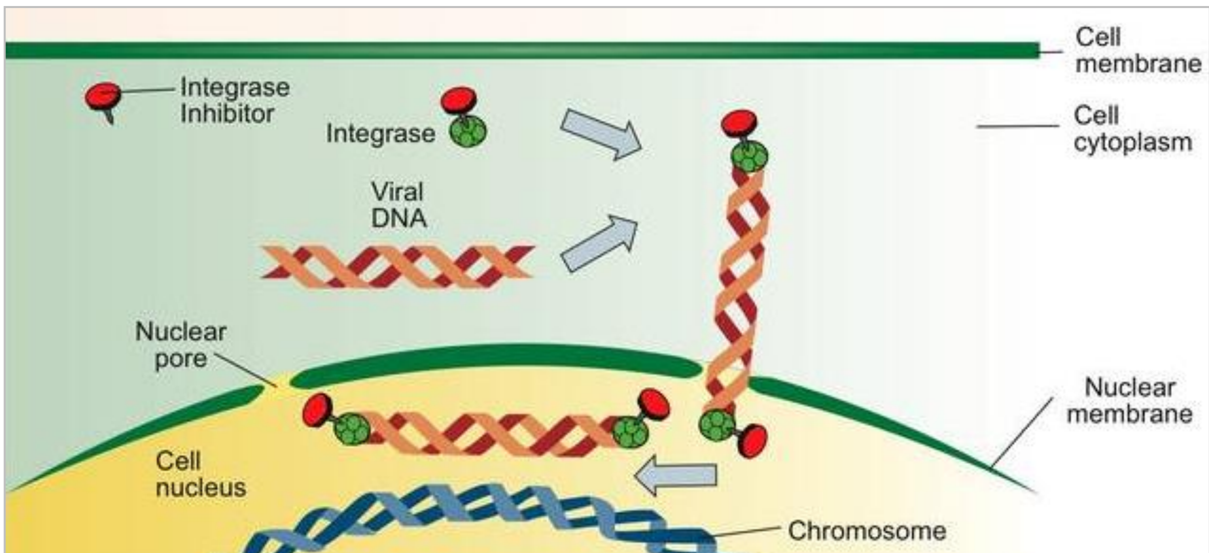

Figure 6 Mechanisms of action of integrase inhibitors

(Reproduced as is from Immunopaedia. Advancing global immunology education. Immunopaedia.org. Retrieved October 2021, from https://www.immunopaedia.org.za/treatment-diagnostics/hiv-infection-treatment/arv-drug-information/)

1.2.4 Antiretroviral therapy in South Africa

South Africa began rolling out antiretroviral drugs in the public health sector in 2004 [73]. Initially provision of ART was practically entirely hospital-based because of accreditation requirements and it was restricted to HIV patients with CD4 counts below 200 cells/µL [73]. By 2010, eligibility was expanded to CD4 counts lower than 350 cells/µL and all pregnant women living with HIV were eligible for life-long ART [73]. Given the benefits of early treatment initiation, the government moved to UTT in 2016, increasing the number of people receiving ART significantly [73]. Up to 2019, South African HIV treatment guidelines recommended use of TDF with 3TC and EFV (TLE) in first-line ART, with PIs reserved for second-line ART, and INSTIs for third-line ART. VL testing is done at ART initiation, at 3, 6, and 12 months, and thereafter annually if the VL remains below a 1,000 copies/mL [69,74]. However, in the case of insufficient viral suppression, intensive adherence counselling with repeat VL testing after 2 months is recommended [74]. If the subsequent VL remains above 1,000 copies/mL (i.e., two consecutive VLs >1,000 copies/mL on ART), the patient is considered as failing first-line treatment and second-line ART is recommended. As with first-line ART, patients with persistent viremia on second-line ART are considered to be failing treatment and only then is genotypic drug resistance testing recommended to select drugs for third-line ART [74]. In 2019, due to increased levels of NNRTI PDR, the South African HIV treatment guidelines adopted use of DTG in first-line and subsequent ART regimens. Table 2 shows a summary of previous and current South African adult ART regimen guidelines.

Table 2. Previous and current first- and second-line regimens for adults living with HIV in South Africa

	First-line regimen		**Second-line regimen**	
	Previous (NNRTI-based)	**Current (INSTI-based)**	**Previous (PI-based)**	**Current (INSTI-based)**
Preferred regimen	TDF + XTC + EFV	TDF + XTC + DTG [a]	AZT + 3TC + LPVr	AZT + 3TC + DTG
Alternative regimen	AZT + 3TC + EFV	TDF + XTC + EFV [b]	TDF + 3TC + LPVr	NRTI + XTC + DTG [c] or TDF + FTC + DRVr
Resistance testing	Not required	Not required	Required	Required

3TC, lamivudine; AZT, zidovudine; DRVr, ritonavir-boosted darunavir; DTG, dolutegravir; EFV, efavirenz; FTC, emtricitabine; INSTI, integrase strand transfer inhibitors; LPVr, ritonavir-boosted lopinavir; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitors; TDF, tenofovir; XTC, lamivudine or emtricitabine

[a] This includes individuals already on first-line ART with VLs <50 copies/mL

[b] If intolerant to DTG

[c] If previously on AZT-based ART, perform resistance testing and use whichever NRTI is fully active with XTC + DTG

1.2.5 HIV drug resistance (HIVDR)

HIVDR mutations are known to reduce susceptibility to ARV drugs. Mutations are named starting with the wild-type amino acid (one letter code), followed by the codon position of the amino acid, and then the mutant amino acid (one letter code). For example, K65R mutation means that lysine (K) has been replaced by arginine (R) at codon position 65 of the RT gene (Figure 7).

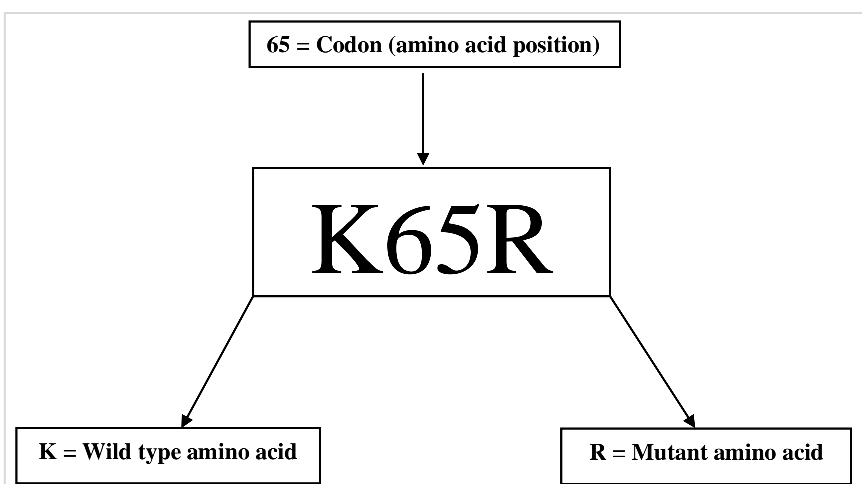

Figure 7 HIV drug resistance mutation nomenclature

There are two main types of HIVDR, namely PDR and ADR. PDR is drug resistance in individuals initiating or re-initiating ART, with or without prior ART exposure [75]. PDR can occur in any of three ways; transmission of drug-resistant HIV from a person with acquired drug resistance (ADR), transmission of primary drug-resistant HIV from another ART-naïve person, or from prior exposure to antiretroviral drugs for treatment or prevention [75,76]. Presence of PDR is associated with poor virological outcomes on first-line ART [77]. Several low and middle-income countries previously reported levels of PDR (largely due to NNRTI-associated resistance mutations) at levels above 10%, leading to the prompt recommendation to replace NNRTIs with DTG in first-line regimens [78]. In contrast to PDR, ADR is the most common type of drug resistance that occurs when HIV continues to replicate in the presence of ARV drugs [79]. This is mainly due to sub-optimal drug concentrations, resulting from poor treatment adherence, treatment interruptions, and inadequate drug metabolism [79]. Generally, more than 70-80% of people with virological failure develop ADR [80].

There are two main methods of HIVDR testing, namely phenotypic and genotypic testing. Phenotypic testing measures rate of viral replication in the presence of ARV drugs [23]. This is done *in vitro* with the ability to detect drug resistance levels or susceptibility of the virus without pre-existing knowledge of the mutations [81]. In phenotypic testing, virus replication at different drug concentrations is monitored by expression of a reporter gene and is compared with the replication of a reference HIV strain (Figure 8) [82]. The drug concentration that inhibits viral replication by 50% which is the median inhibitory concentration [IC_{50}] is calculated (Figure 9), and the ratio of the IC_{50} of test and reference viruses are reported as fold increase in IC_{50} (i.e., fold resistance) [83]. However, phenotypic testing is not common in RLS due to its high cost, lengthy turn-around time, and the requirement for highly specialized facilities [84].

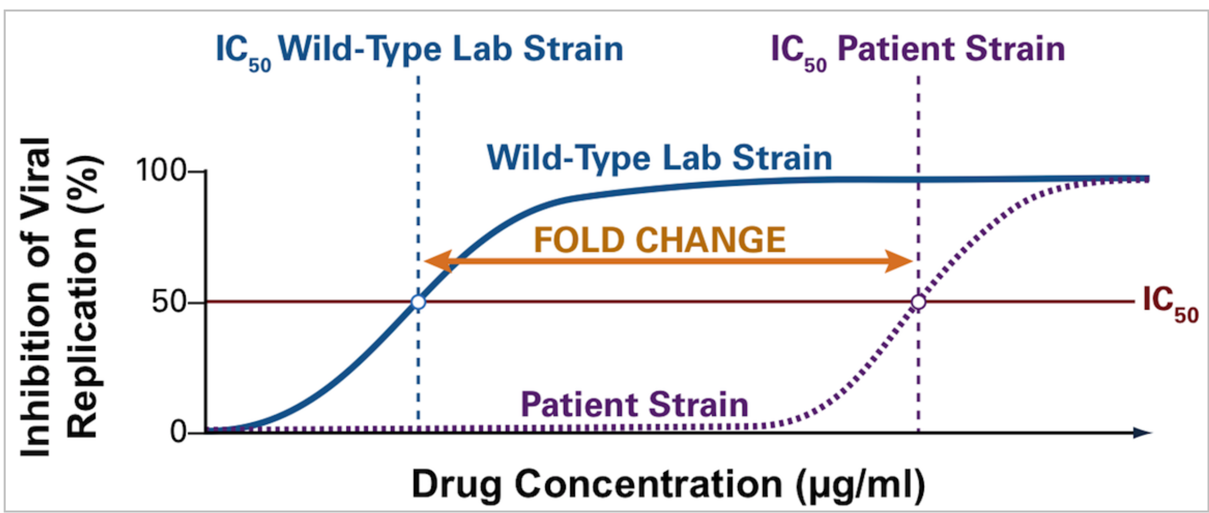

Figure 8 Phenotypic testing inhibitory concentration curve
(Reproduced as is from Spach DH, Kinney RG (2021, October 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved October 2021, from https://www.hiv.uw.edu/go/antiretroviral-therapy/evaluation-management-virologic-failure/core-concept/all#interpretation-drug-resistance-assays)

On the contrary, genotypic testing detects specific HIV genetic sequence changes to determine whether there has been a change in structure compared to 'wild-type' virus (a viral sample with no genetic mutations or drug resistance) [81]. Based on prior knowledge of specific mutations, genotypic testing can be used to predict susceptibility of the virus to ARV drugs [81]. To this date, genotypic testing is the preferred method of HIVDR testing due to the relatively low cost, shorter turn-around time, and detection of genetic mutations which includes mixtures [85]. Common genotypic testing by Sanger

sequencing involves several steps which include plasma or dried blood spot (DBS) sample collection, viral RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR), nested polymerase chain reaction (PCR), gel electrophoresis, PCR product purification, cycle sequencing, sequencing purification, and capillary electrophoresis (Figure 9) [86]. The resulting DNA sequences from each sample are analysed for HIVDR mutations commonly using the Stanford HIV Drug Resistance Database (Stanford HIVdb) [87]. Stanford HIVdb is arguably the most common tool used for monitoring ADR and TDR and in developing new ARV drugs [59]. Researchers focusing on HIVDR also use HIVdb for comparison of their findings to previous studies and to perform meta-analyses.

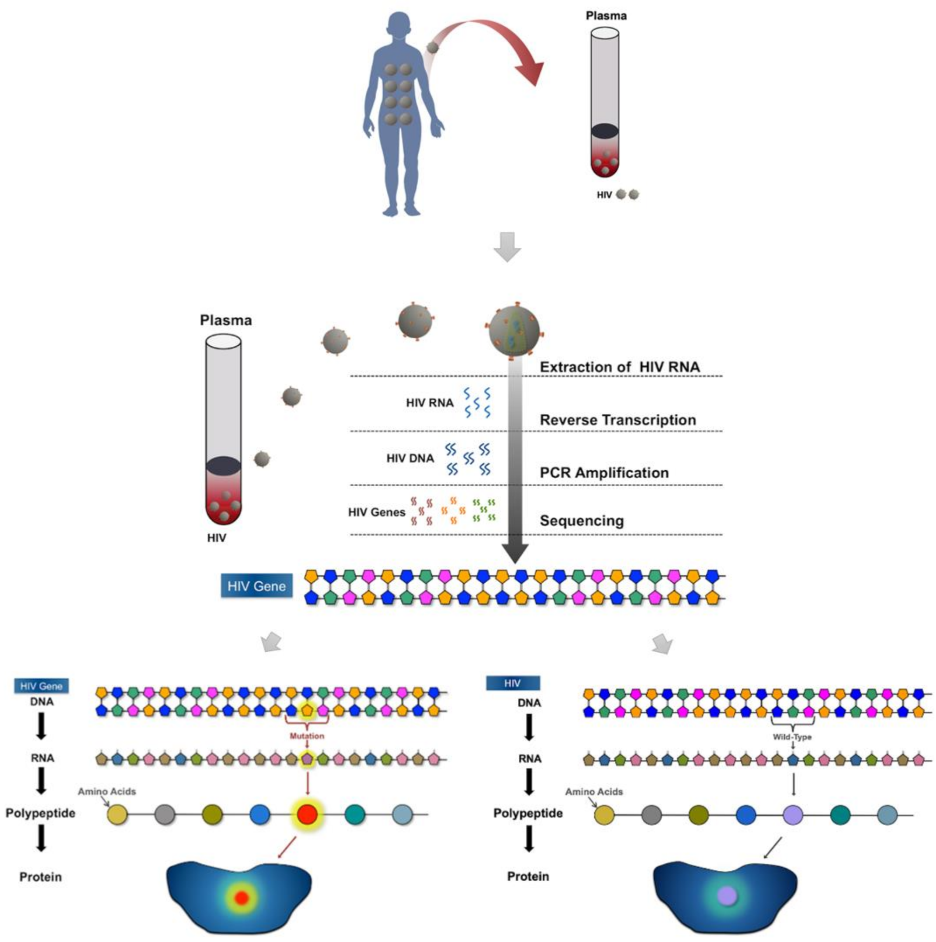

Figure 9 Summary of wet laboratory steps in genotypic HIVDR testing

(Reproduced with modifications from Spach DH, Kinney RG (2021, October 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved October 2021, from https://www.hiv.uw.edu/go/antiretroviral-therapy/evaluation-management-virologic-failure/core-concept/all#interpretation-drug-resistance-assays)

Genotypic resistance testing assays are still considered specialized tests in RLS mainly due to their lack of affordability and scalability [88]. Several point mutation assays (PMAs) have been developed to provide cheaper and faster turn-around times for detecting HIVDR mutations. PMAs detect a limited number of mutations at any given time, hence their utility has been scarce, especially in clinical diagnostics [89].

1.2.6 Point mutation assays

PMAs are low-cost assays that typically use probes to detect specific amino acid changes, and generally have high sensitivity for detecting major and minor variant mutations. However, prior knowledge of targeted mutations is required. PMAs are limited by inability to multiplex samples hence they are labour intensive when processing numerous samples, require wide validations and are not commercially available. The most common PMAs for HIVDR testing target *protease* and *reverse transcriptase* mutations, and thus require 'probe' adjustments for use in detecting IN mutations. These include allele-specific polymerase chain reaction (AS-PCR), pan-degenerate amplification and adaptation (PANDAA), and oligonucleotide ligation assay (OLA) [90].

1.2.7 Allele-specific polymerase chain reaction (AS-PCR)

AS-PCR is a quantitative polymerase chain reaction (qPCR) assay with the ability to quantify and detect minor HIV-1 variants occurring at less than 1% frequencies within the viral pool [91,92]. This highly sensitive assay is significantly less labour-intensive, less time-consuming, and can be used for detecting specific HIVDR mutations in people that have been infected with HIV despite use of pre- and or post-exposure prophylaxis, and following vertical HIV transmission (i.e., mother-to-child-transmission) [93]. However, as most PMAs, this assay is limited by the number of resistance mutations that can be detected simultaneously, because detection of each mutation often requires a separate PCR reaction [76,94]. Also, the assay's sensitivity can be reduced drastically when there is high variability in regions around mutation sites [95].

1.2.8 Pan-degenerate amplification and adaptation (PANDAA)

PANDAA is a qPCR assay that uses degenerate primers to target specific mutation sites, regardless of high HIV diversity around the mutation site [96]. Degenerate primers are favorable in that they have sequences in which positions contain a number of possible bases, thus primers have different nucleotide combinations that can bind to regions with high diversity. PANDAA is designed to identify specific

mutations impacting susceptibility to NRTIs and NNRTIs, namely K65R, K103N, V106M, Y181C, M184V, and G190A, which represent the most common mutations in individuals receiving NNRTI-based first-line regimens and failing treatment [97]. This assay can quantify key HIVDR mutations present at ≥5% of the viral population, with high sensitivity and specificity, and at a low cost [97]. Similar to AS-PCR, an initial investment in a qPCR instrument and molecular laboratory facilities are required, which still limits use of PANDAA in RLS [98].

1.2.9 Oligonucleotide ligation assay (OLA)

On the other hand, OLA is a PMA that does not require a huge investment in laboratory equipment and specialized facilities. OLA is designed to identify specific mutations impacting susceptibility to NRTIs and NNRTIs, namely K65R, K103N, V106MI, Y181C, M184V, and G190A [99,100]. Its DNA amplification procedure uses three probe-ligated primer sets at a temperature that tolerates annealing of sequences with mismatches [100,101]. The more recent OLA-SIMPLE system uses lyophilized reagents reducing the need for cold chain storage, and simplifies detection of HIVDR using a lateral flow assay [102]. Moreover, it is relatively less expensive and has a shorter turnaround time, making it a preferable choice for RLS [103]. In comparison to Sanger sequencing, OLA workflow requires less instrumentation, lower reagent cost, minimal hands-on time, and offers higher sensitivity [100,103].

1.2.10 Sanger sequencing and next generation sequencing (NGS)

Sanger sequencing remains the gold-standard method used in detecting HIVDR mutations for clinical diagnostics (and for research purposes). However, Sanger sequencing does not reliably detect mutations at <20% within the viral population [104]. On the other hand, NGS is rapidly becoming a common sequencing approach, with the ability to detect low-abundance drug-resistant variants (LA-DRVs) at <20% frequency [104]. Moreover, NGS sequencing costs have been reducing given its ability to pool samples and perform massive parallel sequencing. However, the clinical significance of LA-DRVs on ART is still not well understood [88,104]. Similar to most technologies, use of Sanger sequencing and NGS in RLS is mainly limited by infrastructural costs and requires bioinformatic expertise. Details of Sanger sequencing and NGS technologies, and recommendations on implementing HIVDR genotyping in RLS have been described in a peer reviewed manuscript in Chapter 2 of this thesis [88].

1.3 Study Rationale and Justification

Given that ART in RLS has been largely limited to NRTIs, NNRTIs and PIs over the years, most drug resistance testing methods have been justifiably focused on genotyping the HIV PR and RT genes.

However, as ART programs in RLS transition towards use of more potent HIV drugs such as DTG in first-line therapy [105,106], there has been an increasing demand for IN resistance testing. HIVDR testing has been largely limited by costs in RLS, making it less accessible to the general public, as evidenced by lack of HIVDR testing among people initiating and or failing first-line treatment. Moreover, HIVDR tests often have long turn-around times, leading to delayed action on managing patient treatment, and in some cases patients are lost to follow-up. Given these challenges, including IN genotyping as a separate assay to PR and RT genotyping means that for each patient two separate HIVDR testing assays should be performed, which theoretically doubles the workload. The costs and time of genotyping also tends to increase when performing assays that use different reagents, PCR conditions and consumables. Therefore, having a single assay method that assesses HIVDR in all relevant viral genes (PR, RT and IN) is preferable to reduce the cost and time required for HIVDR testing, making genotyping simple, affordable, and accessible in a timely manner to guide treatment decisions.

1.4 Study aim

To design a HIV genotypic testing method for detecting drug resistance mutations in the PR, RT and IN genes.

1.5 Study objectives

1. To design a simple and affordable method for detecting HIVDR mutations in the PR, RT and IN genes.
2. To conduct cost-estimate analysis and estimate clinical benefits of using the designed method.

1.6 Study method and settings

1.6.1 Inclusion and exclusion criteria

This study used remnant plasma samples from a previous cross-sectional study on ADR among HIV-positive adults (≥18 years) accessing routine care at East Boom Community Health Centre in Pietermaritzburg, in the uMgungundlovu district (a HIV hyperendemic setting), in central KZN, South Africa. Participants were eligible to be included in the cross-sectional study if they were documented HIV-1 positive adults receiving first, second- or third-line ART for a period of at least 6 months, with a latest VL ≥1000 copies/mL. Remnant plasma samples collected between May and September 2019 were used for this study at the National Health Laboratory Service (NHLS) Department of Virology (University of KwaZulu-Natal), Inkosi Albert Central Hospital (IALCH), in Durban, South Africa.

Details of the study have been described previously as part of the CAPRISA Advanced Clinical Care Programme, hereafter referred to as CAP103 study [69].

1.6.2 CAPRISA and the Advanced Clinical Care Programme

CAPRISA, was established in 2002 under the NIH-funded Comprehensive International Program of Research on AIDS (CIPRA) and is the South African Department of Science and Innovation and the National Research Foundation‘s designated Centre of Excellence in HIV Prevention. Its goal is to undertake globally relevant and locally responsive research that contributes to understanding HIV, TB and SARS-CoV-2 pathogenesis, epidemiology, prevention, and treatment. CAPRISA conducts research in four main Scientific Programmes namely: HIV and Covid-19 pathogenesis and vaccines; HIV and TB treatment; HIV epidemiology and prevention; and SARS-CoV-2 epidemiology, prevention, and vaccines. The CAPRISA Advanced Clinical Care (ACC) Program addresses capacity building and health systems strengthening in the management of patients with advanced HIV and TB, including ART and TB drug resistance management.

1.6.3 National Health Laboratory Service (NHLS)

The NHLS, established in 2001, is the largest diagnostic pathology service in South Africa, supporting national and provincial health departments in the delivery of healthcare. Its activities comprise of diagnostic laboratory services, research, teaching and training. The Department of Virology (DOV) at Inkosi Albert Luthuli Central Hospital (IALCH) is an Academic Department at NHLS in KZN and the University of KwaZulu-Natal. DOV is a diagnostic laboratory providing routine testing for patients attending IALCH and other surrounding hospitals in KZN. It also plays a significant role in conducting academic laboratory-driven research projects.

DOV has experience in several different testing methods, including cell culture and virus isolation (e.g. HIV tissue culture), viral serology (e.g. Hepatitis B surface antibody quantification, SARS-CoV-2 antibody assays), viral quantitative (e.g. HIV viral load) and qualitative PCR (e.g. respiratory multiplex PCR and, more recently, SARS-CoV-2 PCR assays) and Sanger Sequencing. The molecular laboratory within DOV has several different platforms and instruments, including the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, United States) which is used for HIVDR routine testing on samples referred from all public-sector healthcare facilities in KZN. Thus, DOV is the sentinel laboratory for HIVDR testing in KZN, making it an ideal site to conduct this work.

1.6.4 Sample collection and transport

Remnant plasma samples were retrieved from the CAPRISA biorepository and transported on dry ice to the NHLS Department of Virology, approximately 6 km away. Upon receipt, samples were stored at -80°C, until use.

1.6.5 Ethical approval

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN) (BREC/000026/2021) (Appendix 1). CAP103 letter of support to use remnant samples was obtained from CAPRISA (Appendix 2) and ethical approval for the CAP103 study was also obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE419/17) (Appendix 3). All participants gave written informed consent to participate in CAP103 study, and also gave written informed consent for sample storage and re-use.

1.7 Thesis outline

This thesis comprises an introduction, literature review and justification in chapter 1, study manuscripts in chapters 2 to 4, and an overall synthesis in chapter 5. Manuscript formats and referencing styles used in chapters 2 to 4 are according to specific journal requirements, and if published have been presented in their current publication format.

Chapter 1: Introduction, literature review, and justification
This chapter provides the background and overall literature review on HIV drug resistance and this project.

Chapter 2: Manuscript: “HIV-1 Drug Resistance Genotyping in Resource Limited Settings: Current and Future Perspectives in Sequencing Technologies”
This chapter consists of a review paper on Sanger sequencing and NGS platforms, showing challenges and knowledge gaps of sequencing technologies in HIVDR.

Chapter 3: Manuscript: “Affordable drug resistance genotyping of HIV-1 reverse transcriptase, protease and integrase genes, for resource limited settings”
This chapter presents a manuscript consisting of the methodology and results of this project.

Chapter 4: Manuscript: “Affordable method for genotyping HIV-1 reverse transcriptase, protease and integrase genes: an in-house protocol”

This chapter presents a detailed step-by-step protocol of the methodology used in this project.

Chapter 5: Synthesis of the thesis

The chapter presents a summary of the overall significance of this study, recommendations for policy, as well as recommendations for future research.

CHAPTER 2: HIV-1 DRUG RESISTANCE GENOTYPING IN RESOURCE LIMITED SETTINGS: CURRENT AND FUTURE PERSPECTIVE IN SEQUENCING TECHNOLOGIES

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Author Contributions:

Conceptualization, S.M. and B.C.; investigation, S.M.; resources, B.C.; writing-original draft preparation, S.M.; writing review and editing, L.G., M.P., J.M., K.N. and B.C. visualization, S.M. and B.C.; supervision, B.C.; project administration, S.M.; funding acquisition, B.C.

Review

HIV-1 Drug Resistance Genotyping in Resource Limited Settings: Current and Future Perspectives in Sequencing Technologies

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Abstract: Affordable, sensitive, and scalable technologies are needed for monitoring antiretroviral treatment (ART) success with the goal of eradicating HIV-1 infection. This review discusses use of Sanger sequencing and next generation sequencing (NGS) methods for HIV-1 drug resistance (HIVDR) genotyping, focusing on their use in resource limited settings (RLS). Sanger sequencing remains the gold-standard method for detecting HIVDR mutations of clinical relevance but is mainly limited by high sequencing costs and low-throughput. NGS is becoming a more common sequencing method, with the ability to detect low-abundance drug-resistant variants and reduce per sample costs through sample pooling and massive parallel sequencing. However, use of NGS in RLS is mainly limited by infrastructure costs. Given these shortcomings, our review discusses sequencing technologies for HIVDR genotyping, focusing on common in-house and commercial assays, challenges with Sanger sequencing in keeping up with changes in HIV-1 treatment programs, as well as challenges with NGS that limit its implementation in RLS and in clinical diagnostics. We further discuss knowledge gaps and offer recommendations on how to overcome existing barriers for implementing HIVDR genotyping in RLS, to make informed clinical decisions that improve quality of life for people living with HIV.

Keywords: HIV-1 drug resistance; Sanger sequencing; next generation sequencing; resource limited settings

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1. Introduction

In an effort to end the HIV epidemic by 2030, the UNAIDS 95-95-95 goals aim to ensure that 95% of all people living with HIV know their status, 95% of all people diagnosed receive sustained antiretroviral treatment (ART), and 95% of all people receiving ART have sustainable viral suppression [1,2]. HIV-1 drug resistance (HIVDR) remains a major threat to achieving these targets, particularly in achieving the third target of 95% viral suppression [3]. HIVDR genotyping before ART initiation and at ART failure improves effectiveness of subsequent treatment [4,5], but its use in resource limited settings (RLS) is often restricted by costs. Although Sanger sequencing remains the preferred clinical diagnostic method for HIVDR genotyping, next generation sequencing (NGS) methods are becoming more affordable but their use in the therapeutic management of patients on ART is yet to be established [6].

Sanger sequencing is a population-based method that uses a di-deoxy chain termination chemistry to generate a single consensus sequence representative of the most common bases at each nucleotide position [7]. This, however, means that Sanger sequencing does not reliably detect mutations that are less represented within the viral pool, also known as low-abundance drug-resistant variants (LA-DRVs) [8,9]. Despite that, HIVDR mutations detected by Sanger sequencing have been shown to predict treatment response, making it a reliable method for use in making clinical decisions [10]. On the other hand, NGS is more sensitive, having the ability to detect LA-DRVs (i.e., viral variants at <20%) [11], and enabling quantitative detection of HIVDR mutations [12]. However, there remains a dearth of knowledge around the clinical significance of LA-DRVs on ART effectiveness [13], although a high concordance with Sanger sequencing has been shown in detection of mutations at $\geq$20% frequency [6].

NGS also has the ability for massive parallel sequencing of individual input templates, generating incredible amounts of data per sequencing run [11,14]. Given its high-throughput and cost-efficiency through parallel sequencing and sample pooling, NGS is becoming a more common method for HIVDR genotyping [14,15]. In this review, we discuss the available and emerging Sanger sequencing and NGS methods for HIVDR genotyping, highlighting challenges for their use in RLS and for clinical diagnostics.

2. Overview of Sanger Sequencing and NGS Platforms

Sanger sequencing platforms have largely been dominated by Applied Biosystems, with instruments ranging from 3100- to 3700-series, as well as the SeqStudio (Applied Biosystems, Tustin, CA, USA) instrument [16]. The ABI 3730xl (Applied Biosystems, Tustin, CA, USA) genetic analyzer is the most robust of these instruments with high-throughput, scalability and flexibility. It can sequence fragments up to 900 bp in a single read and can process 96 reactions simultaneously and continuously for several sequencing plates at a time [17]. However, its throughput is nowhere comparable to NGS, which can produce millions of reads from a single sequencing run [18,19]. Moreover, NGS has several sequencing platforms from different manufacturers such as Illumina (Illumina, CA, USA), Thermo Fisher (Thermo Fisher Scientific, Waltham, MA, USA), Pacific Biosciences (PacBio) (PacBio, CA, USA) and Oxford Nanopore Technologies (ONT) (ONT, Oxford, UK). Illumina uses a sequencing-by-synthesis chemistry and is arguably one of the most widely used NGS platforms, offering flexibility in choice of instruments, with varying read-lengths and sequencing outputs [20]. Considering that HIVDR genotypic testing in RLS is mainly targeted on the HIV-1 *pol* gene, Illumina's short read sequencing and lower instrument cost make it a front runner for HIVDR genotyping [21]. Table 1 provides a summary of the popular NGS platforms, highlighting their technical differences.

Table 1. Summary of popular NGS platforms.

Manufacturer	Platforms	Instrument Cost (US$)	Chemistry	Read Length (bp)	Maximum Output (Gb)	Error Rate (%)
Illumina	iSeq 100 MiniSeq MiSeq NextSeq HiSeq NovaSeq	19,900–950,000	SBS	150–300	0.3–6000	0.1
Thermo Fisher	Ion S5 Ion PGM Ion Proton	60,000–149,000	Ion semiconductor	200–400	0.08–15	1

Table 1. *Cont.*

Manufacturer	Platforms	Instrument Cost (US$)	Chemistry	Read Length (bp)	Maximum Output (Gb)	Error Rate (%)
Pacific Biosciences	Sequel II	350,000–750,000	SMRT	60,000	0.5–10	13
	Sequel IIe					
	Sequel					
Oxford Nanopore Technologies	MinIon	1000–25,000	Nanopore	>100,000	10–960	12
	GridION					
	PromethION					

SBS, sequencing-by-synthesis; SMRT, Single Molecule Real-Time; US$, United States Dollars; bp, base pair; Gb, Gigabase. This table was adapted from the following references [12,22].

3. Sanger Sequencing and NGS for HIVDR Genotyping

Several in-house Sanger sequencing assays have been developed over the years for HIVDR genotyping. However, there is currently one commercially available Sanger sequencing assay approved by the US Food and Drug Administration (FDA) for HIVDR genotyping, namely ViroSeq HIV-1 Genotyping kit [23–26]. The kit is relatively expensive, costing approximately US$380 per test, making it less affordable for RLS [27]. This makes laboratories resort to developing low-cost in-house assays, and in some cases modifying commercial assays to reduce costs [28]. A personal view by Inzaule et al. previously estimated that the costs of HIVDR genotyping in-house assays range from US$47.50 to US$155, without labor costs [14], although these could vary further depending on costs of procuring laboratory reagents and consumables.

A collaborative partnership between the Southern African Treatment and Resistance Network (SATuRN) and not-for-profit manufacturers (Life Technologies/Centers for Disease Control and Prevention) previously developed a discounted HIVDR genotyping assay that reliably detects mutations in the HIV-1 protease (PR) and reverse transcriptase (RT) genes from plasma and dried blood spot samples by Sanger sequencing [14,29–31]. Such partnerships are essential to validate in-house HIVDR genotyping assays and make them commercially affordable for RLS. Table 2 shows a summary of published in-house HIVDR genotyping assays targeted for use in RLS.

There is currently only one commercially available NGS assay approved by the FDA in November 2019 for clinical HIVDR genotyping, known as the Sentosa SQ HIV-1 Genotyping Assay [12,50]. This is a standardized, semi-automated and novel deep in vitro sequencing assay developed by Vela Diagnostics (Vela-Diagnostics, Humburg, Germany) for sequencing the combined HIV-1 PR, RT and integrase (IN) genes with minimal expertise [51]. The instrument alone costs ~US$400,000 with a per sample cost of around US$400, making it an expensive option for RLS. All other NGS methods for HIVDR genotyping are based on in-house assays developed to the convenience of the laboratories running the assays. However, most of these in-house assays are labor intensive and require additional quality control measures to lessen the effect of sequencing errors generated by NGS technologies. Figure 1 summarizes the Sanger and NGS workflows from sample to producing a report.

Table 2. In-house HIVDR genotyping assays for use in resource limited settings.

Year	Source	Country	Specimen Type	Vl Threshold	HIV-1 Gene	PMID
2006	Steegan K et al. [32]	Belgium	Plasma	$\geq$500 cp/mL	PR, RT	16375980
2007	Chen JHK et al. [33]	China	Plasma	$\geq$400 cp/mL	PR, RT	17449318
2008	Van Laethem K et al. [34]	Belgium	Plasma	NS	IN	18706932
2008	Pillay V et al. [35]	South Africa	Plasma	NS	PR, RT	18575198
2009	Hearps AC et al. [36]	Australia	Plasma	>50 cp/mL	IN	19917199
2009	Saravanan et al. [37]	India	Plasma	>1500 cp/mL	PR, RT	19490976
2010	Wallis CL et al. [38]	South Africa	Plasma	>1000 cp/mL	PR, RT	19917318
2010	Yang C et al. [39]	USA	DBS	$<$400 cp/mL	PR, RT	20660209
2011	Zhou Z et al. [30]	USA	Plasma and DBS	$<$400 cp/mL	PR, RT	22132237
2011	Fokam J et al. [40]	Cameroon	Plasma	>1000 cp/mL	PR, RT	21465085
2012	Chen JHK et al. [41]	Hong Kong	Plasma	$\geq$400 cp/mL	PR, RT	22302906
2012	Parkin N et al. [42]	USA	DBS	$\geq$1000 cp/mL	PR, RT	22544187
2013	To SWC et al. [8]	Hong Kong	Plasma	$\geq$1000 cp/mL	IN	23886504
2013	Charturbhuj DN et al. [43]	India	Plasma	$\geq$1000 cp/mL	PR, RT	23353551
2013	Aitken SC et al. [44]	Netherlands	Plasma and DBS	$\geq$1000 cp/mL	PR, RT	23536405
2013	Inzaule S et al. [24]	Kenya	Plasma and DBS	$\geq$1000 cp/mL	PR, RT	23224100
2014	Acharya A et al. [45]	India	Plasma	$\geq$1000 cp/mL	PR, RT	25157501
2014	Manasa J et al. [29]	South Africa	Plasma	$\geq$1000 cp/mL	PR, RT	24747156
2014	Chaturbhuj DN et al. [43]	India	Plasma	>1000 cp/mL	PR, RT	24533056
2015	Armenia D et al. [46]	Italy	Plasma	>50 cp/mL	IN	25712318
2017	Gupta S et al. [47]	Canada	Plasma	>100 cp/mL	PR, RT	28473986
2019	Seatla KK et al. [48]	Botswana	Plasma	>1000 cp/mL	IN	31751353
2020	Chrysostomou AC et al. [49]	Cyprus	Plasma	$\geq$1000 cp/mL	PR, RT, IN	32061896

cp/mL, copies per microliter; DBS, dried blood spots; IN, integrase; NS, not stated; PR, Protease; RT, reverse transcriptase; SA, South Africa; VL, viral load.

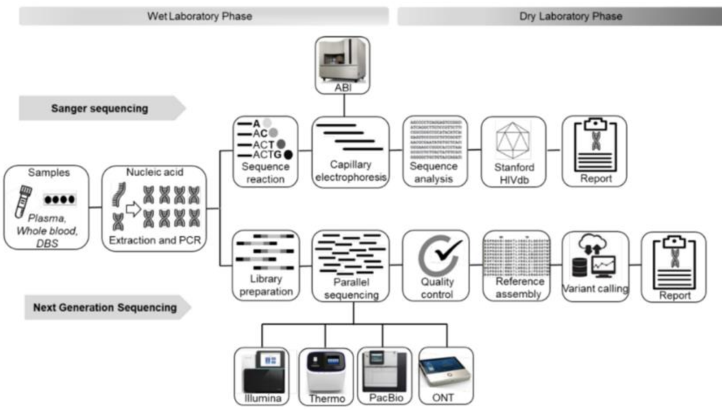

Figure 1. Summary comparison of Sanger sequencing and NGS HIVDR workflows.ABI, Applied Biosystems; DBS, dried blood spots; HIV db, HIV drug resistance database; ONT, Oxford Nanopore Technology; PacBio, Pacific Biosciences; PCR, polymerase chain reaction; Thermo, Thermo Fisher.

4. Advantages and Disadvantages of Sequencing Methods for HIVDR Genotyping

For over two decades Sanger sequencing has been shown to be a highly reproducible and interpretable method for diagnosing HIVDR in clinical settings [14,32,33]. This is because Sanger sequencing has been available long enough for use in HIVDR genotyping, producing high quality sequence data, with short turnaround times, and relatively simple workflows and data interpretation [14]. However, in addition to the inability of Sanger sequencing to reliably detect LA-DRVs [19,52], it has limited data throughput [53].

Also, there is less flexibility in choice of sequencing platforms, which is mainly limited to one manufacturer (i.e., Thermo Fisher). Moreover, Sanger sequencing requires standard molecular biology workflows and infrastructure which are mostly available in centralized laboratories in RLS [14]. This reduces its accessibility and affordability for HIVDR genotyping in RLS; hence, its preferred for use only after second-line ART failure [54].

In contrast, there is a wider range of NGS platforms supplied by various manufacturers, using different sequencing chemistries (Table 1) and achieving massive parallel sequencing [8]. This offers laboratories flexibility in choosing which assays to run but poses a challenge in reproducibility across various platforms. However, there is lack of validated NGS methods for clinical use, which is compounded by high sequencing errors and workflows that are labor intensive. NGS methods also require high infrastructure costs and high levels of expertise [14]. In addition, long-term investment in remote and/or cloud-based servers is required to keep up with the large amounts of data generated by NGS. The vast amounts of data also pose challenges in data analysis [55]. Individual laboratories often develop their own data analysis pipelines, making it difficult to normalize data quality across the board. Table 3 summarizes the strengths and weaknesses of Sanger sequencing and NGS in relation to their use in HIVDR genotyping [14].

Table 3. Strengths and limitations of Sanger sequencing and NGS in HIVDR genotyping methods.

	Strengths	Weaknesses
Sanger sequencing	• Several validated methods for clinical use • Low sequencing errors • Relatively simple workflows and data analysis • Relatively shorter turnaround times • Common method in RLS • Easily accessible software for interpretation (such as Stanford HIVdb)	• High cost per test • Cannot reliably detect LA-DRVs • Not suitable for sequencing long genes/large genomes • Not suitable for parallel testing • High infrastructure costs • Require standard molecular biology workflows
Next generation sequencing	• Lower cost per test through pooling • High sensitivity for LA-DRVs • Suitable for sequencing long genes/large genomes • Massive parallel sequencing	• Only one validated method for clinical use (Sentosa HIV-1 genotyping kit) • High sequencing errors • Complex labor-intensive workflows and data analysis • Longer turnaround time • High infrastructure costs • Requires specialized facilities • No clear clinical significance of LA-DRVs • Can produce unequal sequencing coverage • Software for interpretation depend on data output files • Requires personnel with high-level expertise • Require standard molecular biology workflows

RLS, resource limited settings; DBS, dried blood spots; LA-DRV, low-abundance drug-resistant variants. This table was adapted from the following reference [14].

5. Future Recommendations for HIVDR Genotyping and Knowledge Gaps

As new drugs to treat HIV-1 patients increase and extensive drug class resistance grows, HIVDR genotyping methods need to evolve to determine resistance to newer drug classes such as capsid inhibitors, entry inhibitors, nucleoside analogue reverse transcriptase translocation inhibitors and Rev inhibitors. For instance, as ART programs in RLS adopt use of integrase strand transfer inhibitors such as dolutegravir and possibly the long-acting injectable cabotegravir, sequencing of longer reads that cover all relevant HIV-1 viral genes (i.e., PR, RT, and IN) is required. Combined PCR amplification of these genes is difficult as

they are spread apart. Genotyping PR and RT genes in a separate assay to the IN gene is an option; however, this doubles the HIVDR genotyping workload. Therefore, there is a need to design Sanger sequencing assays that detect all relevant mutations in a single amplicon, in order to simplify HIVDR genotyping for clinical diagnostics and research use in RLS.

Although there are several advantages of Sanger sequencing over NGS for clinical diagnostic use, further technical innovations are still required to make Sanger sequencing affordable and available in RLS. The high cost per sample remains a major drawback for implementation of HIVDR genotyping in RLS. NGS can reduce the per sample costs through sample pooling which, however, results in longer turnaround times. Centralizing NGS for HIVDR genotyping could help address the challenge of longer turnaround times and higher per sample costs. Subsidizing of instrument and consumable costs by manufacturers for RLS, together with governmental support will also go a long way in reducing costs and establishing NGS in RLS.

Requirements for expert bioinformatics analyses and high sequencing errors also remain major barriers for use of NGS in HIVDR genotyping. There is a need for international standards that guide NGS for HIVDR genotyping, as well as standardized data analysis pipelines to ensure reproducible, accurate and high-quality data outputs. This could be achieved through internal and external quality assurance programs [11], and validating in-house assays against those that are FDA approved, such as the Sentosa SQ HIV-1 Genotyping Assay. Moreover, validation of commonly used data analysis pipelines for HIVDR mutation calling, such as HyDRA, PASeq and MiCall, is required to address challenges with bioinformatics and data quality [12]. These data analysis pipelines commonly comprise filtering of low-quality data, detection and quantification of amino acid variants at known mutation positions.

HyDRA, PASeq and MiCall are freely available web-based data analysis pipelines that require only minimal bioinformatics expertise and infrastructure [55]. They all use the Stanford HIV drug resistance database (Stanford HIVdb) algorithm, allowing for easy HIV resistance interpretation [21]. HyDRA is compatible with both Illumina and Ion torrent sequencing data and it uses quality control and sequencing error models for data quality assurance [21]. Unlike HyDRA, PASeq is compatible with only Illumina sequencing data. It uses quality control, contamination control and APOBEC hypermutation detection for data quality assurance [21]. Similar to PASeq, MiCall is only compatible with Illumina sequencing data. It uses quality control and sequencing error models for data quality assurance [21]. Despite the minor differences in procedures for data processing and reporting, these analysis pipelines have shown comparable HIV resistance interpretation with less bias for variants at $\geq$5% threshold, although the clinical relevance of LADRVs (i.e., including those at $\geq$5%–<20% thresholds) require further investigations [21,55]. Therefore, these data analysis pipelines can drastically reduce quality control problems associated with NGS and have potential to produce consistent HIV resistance data that is easily and rapidly interpreted to inform clinical decisions.

In addition, understanding the clinical relevance of LA-DRVs detected by NGS remains elusive for use of NGS data in HIV-1 treatment clinical decisions [6]. The Sentosa HIV-1 Genotyping Assay detects up to 10% variant threshold, but the decision on which threshold accurately predicts treatment response is yet to be made, and requires international efforts [12]. As NGS platforms continue to improve with reduced sequencing error rates, the ability to sequence multiple samples in a single sequencing run and possibly automating data analysis will make NGS more feasible, accessible and affordable for HIVDR genotyping in RLS [21].

6. Conclusions

HIVDR remains the greatest barrier to sustainable viral suppression on ART, whilst cost remains the greatest barrier to HIVDR genotyping in RLS. Available in-house and commercial sequencing assays should aim to offer accessible and relevant cost-effective HIVDR genotyping that can be used to make clinical decisions. In this review, we emphasize

the need for Sanger sequencing assays to adapt to dynamic HIV-1 treatment programs, to simplify and make HIVDR genotyping affordable. We also highlight that NGS has great potential to achieve low-cost HIVDR genotyping useful for individual and diagnostic public-health use in RLS. Moreover, validation of wet-lab processes and data analysis pipelines is required for optimal detection and consistent interpretation of HIV resistance data for clinical utility. Ultimately, firm commitments and partnerships between the molecular diagnostics industry, local governments in RLS and global health agencies, are required to overcome HIVDR genotyping barriers that have often slowed down efforts by the UNAIDS to end the HIV epidemic [55].

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The manuscript in chapter 2 gave an overview of Sanger sequencing and NGS platforms, sequencing chemistries, challenges, implementation and current knowledge gaps that exist in the field of HIVDR for RLS. It also emphasizes the need to design Sanger sequencing assays that sequence all relevant HIV-1 genes (i.e. PR, RT and RT) in a single amplicon, in order to simplify HIVDR genotyping for clinical diagnostics and research use in RLS. This motivated work presented in chapter 3, based on a HIVDR method that we developed for genotyping all relevant HIV-1 genes. This is a simple and affordable method with relatively short turnaround time, that does not require additional infrastructure for Sanger sequencing, making it suitable for use in laboratories that are already equipped for sequencing in RLS. The manuscript supplementary material is provided in Appendix 4 and figures on Appendix 5.

CHAPTER 3: AFFORDABLE DRUG RESISTANCE GENOTYPING OF HIV-1 REVERSE TRANSCRIPTASE, PROTEASE AND INTEGRASE, FOR RESOURCE LIMITED SETTINGS

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Affordable drug resistance genotyping of HIV-1 reverse transcriptase, protease and integrase genes, for resource limited settings

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36 ## Abstract

37 **Background**: As use of dolutegravir (DTG) becomes more common in resource
38 limited settings (RLS), the demand for integrase resistance testing is increasing.
39 Affordable methods for genotyping all relevant HIV-1 *pol* genes (i.e., *protease* (PR*),*
40 *reverse transcriptase* (RT) *and integrase* (IN)) are required to guide choice of future
41 antiretroviral therapy (ART). We designed an in-house HIV-1 drug resistance (HIVDR)
42 genotyping method that is affordable and suitable for use in RLS.

43 **Methods:** We obtained remnant plasma samples from CAPRISA 103 study and
44 amplified HIV-1 PR, RT and IN genes, using an innovative PCR assay. We validated
45 the assay using remnant plasma samples from an external quality assessment (EQA)
46 programme. We genotyped samples by Sanger sequencing and assessed HIVDR
47 mutations using the Stanford HIV drug resistance database. We compared drug
48 resistance mutations with previous genotypes and calculated method cost-estimates.

49 **Results**: From 96 samples processed, we obtained sequence data for 78 (81%), of
50 which 75 (96%) had a least one HIVDR mutation, with no major-IN mutations
51 observed. Only one sample had an E157Q INSTI-accessory mutation. When
52 compared to previous genotypes, 18/78 (23%) had at least one discordant mutation,
53 but only 2/78 (3%) resulted in different phenotypic predictions that could affect choice
54 of subsequent regimen. All CAPRISA 103 study sequences were HIV-1C as confirmed
55 by phylogenetic analysis. Of the 7 EQA samples, 4 were HIV-1C, 2 were HIV-1D, and
56 1 was HIV-1A. Genotypic resistance data generated using the IDR method were 100%
57 concordant with EQA panel results. Overall genotyping cost per sample was estimated
58 at ~US$43, with a processing time of ~2 working days.

59 **Conclusions**: We successfully designed an in-house HIVDR method that is suitable
60 for genotyping HIV-1 PR, RT and IN genes, at an affordable cost and shorter

61 turnaround time. This HIVDR genotyping method accommodates changes in ART
62 regimens and will help to guide HIV-1 treatment decisions in RLS.

63

64 **Keywords**: HIV-1, pol gene, affordable genotypic testing, drug resistance, resource
65 limited settings, primary integrase resistance

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92 **Background**

93 HIV-1 drug resistance (HIVDR) remains one of the greatest threats to achieving
94 sustainable viral suppression using antiretroviral therapy (ART). HIVDR is largely
95 driven by inadequate viral suppression in individuals on ART, resulting mainly from
96 poor treatment adherence [1,2]. Following ART scale-up, there have been global
97 concerns over increasing levels of transmitted HIVDR [3]. Most studies have shown
98 increasing levels of pre-treatment HIV-1 drug resistance (PDR) mainly driven by non-
99 nucleoside reverse transcriptase inhibitor (NNRTI) mutations [4,5], with a modelling
100 study by Phillips et al., showing a benefit of changing first-line ART regimens in a
101 setting of high NNRTI resistance [6]. This resulted in the World Health Organization
102 (WHO) recommendation for use of dolutegravir (DTG) in all ART regimens [7], given
103 DTG's better tolerability, less adverse effects, higher genetic barrier to resistance, and
104 availability in a fixed dose combination with tenofovir (TDF) and lamivudine (3TC), i.e.
105 TLD.

106

107 Preliminary findings from NADIA and ARTIST trials have shown adequate viral
108 suppression rates among individuals switching to second-line DTG-based ART,
109 regardless of pre-existing resistance at 96-weeks and 48-weeks follow-up,
110 respectively [8–10]. However, the ADVANCE study showed lower viral suppression
111 rates among individuals on DTG-based ART at 96-weeks, raising concerns over long-
112 term treatment outcomes in a setting with high NNRTI-PDR [11]. HIVDR mutations
113 can be detected by genotypic testing to predict drug susceptibility [12], and
114 understanding INSTI primary resistance prior to extensive DTG-rollout remains
115 important. However, routine HIVDR testing is not available to the majority of people
116 living with HIV in RLS due to the high costs of genotyping and the need for specialized

117 facilities [13,14]. The REVAMP study showed some benefit to genotypic resistance
118 testing for failure on first-line NNRTI-based ART prior to drug selection, and a cost-
119 benefit effect from the study is yet to be shown [15]. In South Africa, genotypic
120 resistance testing is recommended at virologic failure on PI or INSTI-based regimens
121 to inform ART drug selection [16].
122
123 With more studies reporting cases of DTG resistance [17–19] and with increased
124 access to integrase strand transfer inhibitors (INSTIs), the demand for INSTI
125 resistance testing has increased and there exists a risk of emergence of increasing
126 INSTI resistance-associated mutations in RLS [20]. Most available HIVDR genotypic
127 methods in RLS entail two separate assays for the detection of HIVDR mutations in
128 the *reverse transcriptase* (RT) and *protease* (PR) genes, and the *integrase* (IN) gene,
129 such as the Applied Biosystems HIV-1 Genotyping kit [21]. In-house assays have been
130 widely used for HIVDR genotyping especially in RLS where cost remains a major
131 barrier. Reducing the cost of HIV-1 genotypic testing through affordable in-house
132 assays and monitoring strategies could improve access [22], which will subsequently
133 improve clinical decisions and treatment outcomes.
134
135 Therefore, we designed an affordable in-house HIV-1 drug resistance testing method
136 for genotyping all relevant HIV-1 *pol* genes (i.e. PR, RT and IN) using a one-step
137 reverse transcription polymerase chain reaction (PCR) and nested PCR on remnant
138 plasma samples.
139
140
141

142 **Methods**

143 We optimized reverse transcriptase and nested PCR for amplification of viral RNA from
144 samples with viral loads (VLs) ≥1,000 copies/mL (i.e. lower limit of detection); the VL
145 used to determine ART failure in RLS [23]. We obtained stored remnant plasma
146 samples from a CAPRISA 103 study (hereafter referred to as CAP103), which was a
147 cross-sectional study aimed at determining prevalence of acquired drug resistance
148 and subsequent susceptibility to DTG-based regimens, among ART-experienced
149 individuals with virologic failure at the East Boom Community Health Care Center, in
150 Pietermaritzburg, South Africa. Participants from CAP103 gave written informed
151 consent for sample storage and for use of their stored samples in future studies.
152 Sequencing in CAP103 was done using an Applied Biosystem HIV-1 Genotyping kit
153 for the PR and RT genes only, with no IN sequencing in all except one participant.
154 Details of the CAP103 study have been published previously [24]. Aliquots of the same
155 samples were processed using the designed in-house method, hereafter referred to
156 as the IDR method.

157

158 We also obtained 7 remnant samples from an external quality assessment (EQA)
159 programme in which the National Health Laboratory Services (NHLS) Department of
160 Virology participates annually to ensure laboratory proficiency testing and competence
161 in HIVDR genotyping, according to ISO 15189 standards. The EQA panel received
162 from the Quality Centre for Molecular Diagnostics (QCMD) consisted of plasma
163 specimens with HIV VLs ≥1,000 copies/mL. The NHLS Department of Virology scored
164 100% in the HIVDR genotyping QCMD assessment. Aliquots of the same samples
165 were genotyped and compared to EQA panel results for IDR method validation.

166 **Laboratory methods**

167 **Viral RNA extraction and polymerase chain reaction**

168 We retrieved remnant plasma samples from -80°C freezer and left them to 169 equilibrate to room temperature prior to processing. In summary, we extracted viral 170 RNA from 500µl of plasma using a NucliSENS easyMAG automated extraction 171 platform (bioMérieux, Marcy l'Etoile, France), according to manufacturer's 172 instructions. We eluted each viral RNA sample in 25µl volume. We performed 173 complimentary DNA synthesis and first-round PCR on a ProFlex PCR System (Applied 174 Biosystems, Foster City, United States), to amplify an ~4kb HIV-1 *pol* region using 175 SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, Waltham, US). 176 Forward primer PANA2AF (GAGGCAATGAGCCAARCAAACA, HXB2: 1882 - 1903) 177 and reverse primer PANA3AR (TTCCAGGGCTCTAGKTTAGG, HXB2: 5846 - 5865) 178 were used in One-Step RT-PCR. For each sample, we added 5µl of RNA for a total 179 25µl reaction volume, and included a negative control (5µl of nuclease free water) in 180 each PCR. Details of PCR and amplification conditions are shown in Table 1.

181

182 **Table 1** Reverse transcription and first-round PCR conditions

Reagent		**Volume per reaction (µl)**	**Concentration per reaction**
2X Reaction RT-PCR master mix		12.5	1X
Nuclease-free Water		2.25	-
PANA2AF (5µM)		2.5	0.5µM
PANA3AR (5µM)		2.5	0.5µM
SSIV/ Platinum SuperFi DNA polymerase (2X)		0.25	0.02X
Total volume		**20**	-
Thermocycling conditions			
	Temperature (°C)	**Time**	**Cycle(s)**
cDNA synthesis	50	10 minutes	1
Pre-denaturation	98	2 minutes	1
Denaturation	98	10 seconds	
Annealing	56	20 seconds	40
Extension	72	2 minutes	
Final extension	72	10 minutes	1
Hold	4	∞	Hold

183 cDNA, complimentary DNA; RT-PCR, reverse transcription polymerase chain reaction; SSIV, 184 SuperScript IV enzyme; µl, microliter; µM, micromolar; °C, Degrees Celsius

185

186 We performed second-round nested PCR on a ProFlex PCR System (Applied
187 Biosystems, Foster City, United States), using Platinum *Taq* DNA Polymerase
188 (Thermo Fisher Scientific, Waltham, US). Forward primer Pro1 (TAGAGCCAACAGCC
189 CCACCA, HXB2: 2147 -2166) and reverse primer 5066R (ATCATCACCTGCCATCT
190 GTTTTCCAT, HXB2: 5041 - 5066) were used in the nested PCR. For each sample,
191 we added 2µl of first-round amplicon for a total 25µl reaction volume. We verified
192 successful amplification of an ~2.9kb amplicon on 1% agarose gel. Details of second-
193 round PCR and amplification conditions are shown in Table 2.

194

195 **Table 2** Second-round PCR conditions

Second-round PCR Mastermix			
Reagent		**Volume per reaction (µl)**	**Concentration per reaction**
Nuclease-free Water		18.4	-
10X PCR Buffer		2.5	1X
$MgCl_2$ (50mM)		1.0	2mM
dNTP (10mM)		0.5	0.2mM
Pro1 (5µM)		0.25	0.05µM
5066R (5µM)		0.25	0.05µM
Platinum *Taq* DNA Polymerase		0.1	-
Total volume		**23**	-
Thermocycling conditions			
	Temperature (°C)	**Time**	**Cycle(s)**
Pre-denaturation	94	2 minutes	1
Denaturation	95	10 seconds	
Annealing	56	20 seconds	40
Extension	72	2 minutes	
Final extension	72	10 minutes	1
Hold	4	∞	Hold

196 dNTP, deoxynucleoside triphosphate; $MgCl_2$, magnesium chloride; mM, millimolar; µl, microliter; µM,
197 micromolar; °C, Degrees Celsius
198

199 For any sample that failed amplification, we designed a two-fragment approach to
200 amplify the PR, RT and IN genes separately. Details of primers, and PCR conditions
201 for the two-fragment approach are shown in Supplementary Tables S1 – S4.

202 **PCR product purification and Sanger sequencing**

203 For each successfully amplified sample, we performed PCR product purification by
204 incubating 10µl of amplicon with 4µl of ExoSAP-IT Express PCR Product Cleanup
205 reagent (Thermo Fisher Scientific, Waltham, US) at 37°C for 4 minutes and 80°C for
206 1 minute, with a hold at 4°C. We performed cycle sequencing using BigDye Terminator
207 v3.1 kit (Applied Biosystems, Foster City, CA, US) and sequence reaction purification
208 using BigDye XTerminator v3.1 purification kit (Applied Biosystems, Foster City, CA,
209 US), as described previously [25]. We sequenced samples on an ABI 3730 Genetic
210 Analyzer (Applied Biosystems, Foster City, United States) with 8 sequencing primers
211 designed to cover complete HIV-1 PR, RT and IN genes. Four primers covered PR
212 (codons 1 to 99) and RT (codons 1 to 560) genes, and four primers covered the IN
213 gene (codons 1 to 288). Details of sequencing primers used are shown in Table 3.
214 Primers used in this study were obtained from research articles published previously
215 [25–27].

216

217 **Table 3** Sequencing primers for complete HIV-1 PR, RT and IN sequencing

Primer (Direction)	Primer sequence	HXB2	Gene
RTC1F (Forward)	ACCTACACCTGTCAACATAATTG	2486 - 2508	PR and RT
RTC2R (Reverse)	TGTCAATGGCCATTGTTTAACCTTTGG	2630 - 2604	PR and RT
RTC3F (Forward)	CACCAGGGATTAGATATCAATATAATGTGC	2965 - 2994	PR and RT
RTC4R (Reverse)	CTAAATCAGATCCTACATACAAGTCATCC	3101 - 3129	PR and RT
KVL076 (Forward)	GCACAYAAAGGRATTGGAGGAAATGAAC	4161 - 4188	IN
KVL082 (Forward)	GGVATTCCCTACAATCCCCAAAG	4647 - 4669	IN
KVL083 (Reverse)	GAATACTGCCATTTGTACTGCTG	4750 - 4772	IN
PAN2R (Reverse)	CTGCCATCTGTTTTCCATAYTC	5037 - 5058	IN
Optional primers			
2586F (Forward)	AAGCCAGGAATGGATGGCCCA	2586 - 2606	PR and RT
2713R (Reverse)	GGATTTTCAGGCCCAATTTTTG	2713 - 2692	PR and RT
PAN3F (Forward)	TTAAAAGAAAAGGGGGGATTGGG	4783 - 4805	IN
KVL084 (Reverse)	TCCTGTATGCARACCCCAATATG	5243 - 5265	IN

218 HXB2, nucleotide position of HIV-1 reference sequence; IN, integrase; PR, protease; RT, reverse
219 transcriptase
220

221 **Sequence analysis and phylogenetics**

222 Following capillary electrophoresis, we performed sequence editing using Geneious
223 Prime software 2021.1.1 (Biomatters Ltd, New Zealand) [28]. We assessed HIVDR
224 mutations using the Stanford University HIV drug resistance database (version 9.0)
225 [29]. We excluded sequences without complete PR and RT genes. Complete PR and
226 RT gene sequence pairs were evaluated for differences between the IDR method and
227 CAP103. We predicted subsequent ART regimens in discordant sequence pairs based
228 on South African national ART guidelines and previous research evidence [16].

229

230 For phylogenetic analysis, we combined all sequence pairs and included HIV-1
231 reference sequences obtained from the Los Alamos Database (hiv.lanl.gov). We
232 aligned sequences in Geneious software using ClustalW and trimmed sequences to
233 compare similar gene regions between sequence pairs. We performed maximum
234 likelihood tree reconstruction using a generalized time reversible model with proportion
235 of invariable sites and gamma distribution (GTR + I + G), and with 100 bootstrap
236 replicates.

237

238 For IDR method validation, we compared IDR sequence data to corresponding gene
239 regions of EQA sequences generated at the NHLS Department of Virology. HIV-1
240 subtype classification of EQA sequences was determined from the Stanford HIV drug
241 resistance database and confirmed using the REGA HIV-1 Subtyping Tool [30].
242 Detailed steps of the IDR method are available on protocols.io,
243 dx.doi.org/10.17504/protocols.io.b5tvq6n6.

244

245 **Cost-estimate analysis**

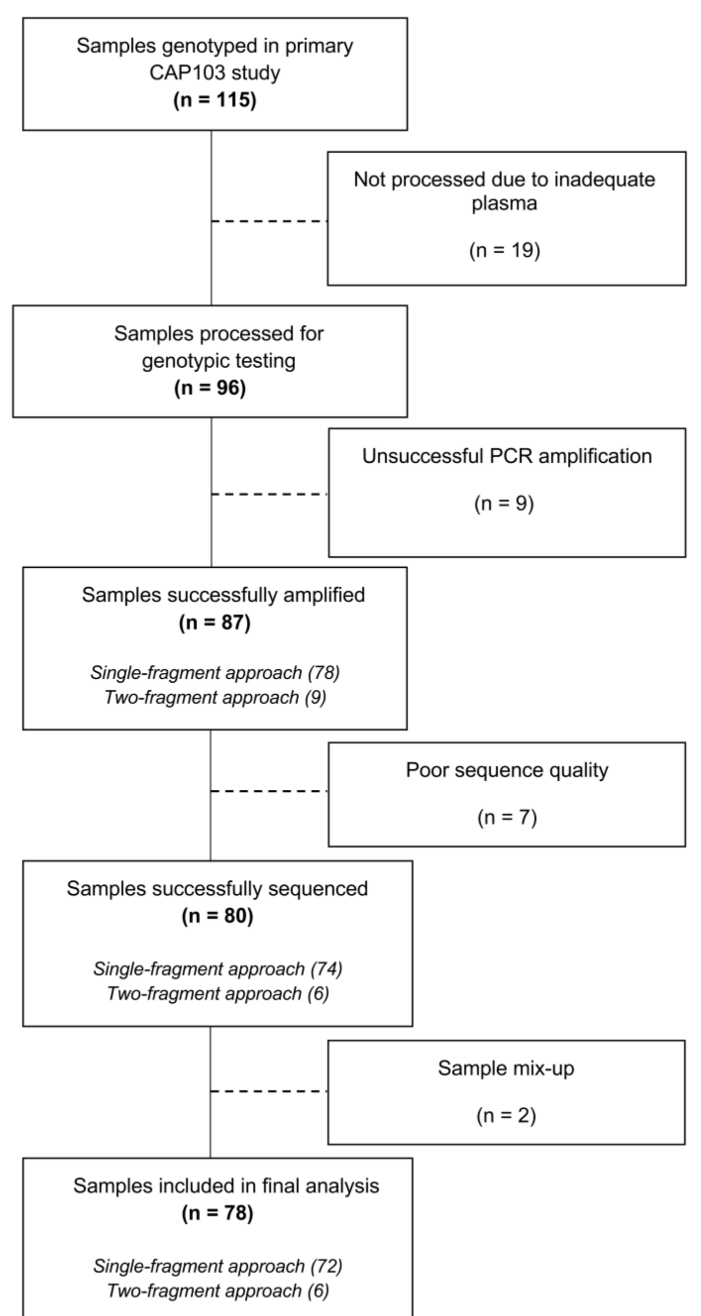
246 We performed a cost breakdown of consumables required for the IDR method using
247 pricing from product catalogues, and estimated genotyping turnaround time. At the
248 time of analysis we used an exchange rate of $1 US Dollar to ZAR15 South African
249 Rands. In addition, we estimated the cost of genotyping using the alternative two-
250 fragment approach.

251

252 ## Results

253 Overall, 115 remnant plasma samples with previous HIV-1 genotype results were
254 obtained, and 19 samples were not processed due to inadequate plasma (i.e. plasma
255 volume <500µl available for extraction). Of 96 samples processed, we included 78
256 (81%) samples with complete PR, RT and IN sequences in final analysis (Figure 1). A
257 complete list of all samples processed and their sequence outcomes are summarized
258 in Supplementary Table S5.

259



260

261 **Figure 1** Summary flow chart of samples in IDR study from request to analysis

262 **Amplification and sequencing**

263 Overall, 87 of 96 (91%) samples were successfully amplified, 78 (90%) as a single
264 amplicon of the PR, RT and IN genes, with an additional 9 samples amplified using
265 the two-fragment approach (i.e. PR and RT, and a separate IN amplicon). The median
266 VL of samples included in final analysis were higher compared to samples which failed
267 amplification, i.e. median VL 4.4 $\log_{10}$ copies/mL, (interquartile range (IQR): 3.8 – 4.9)
268 vs. 3.7 $\log_{10}$ copies/mL (IQR: 3.2 – 4.7), p = 0.06 (Wilcoxon rank-sum (Mann Whitney)
269 test). A representative gel image of complete *pol* gene amplification is shown in
270 Supplementary Figure S1. Sequence coverage of the complete *pol* gene was achieved
271 using 8 sequencing primers as shown in Figure 2.

272

273

274 **Figure 2** Complete sequence coverage of PR, RT and IN genes with 8 sequencing primers

275 **HIV drug resistance mutations and sequence comparison**

276 Of 87 samples successfully sequenced, two (IDR074 and IDR076) were excluded due
277 to potential sample mix-up, and 7 had poor sequence quality. Of 78 sequences
278 included in final analysis, 75 (96%) had at least one drug resistance mutation in either
279 PR and RT genes, similar to CAP103. There were no major IN drug resistance
280 mutations observed. Only one sample (IDR113) had an IN accessory mutation (i.e.
281 E157Q). We observed similar proportions of protease inhibitor (PI), nucleoside reverse
282 transcriptase inhibitor (NRTI), and NNRTI resistance mutations when compared to
283 previous CAP103 genotypes. The most common PI drug resistance mutation was
284 M46I, occurring in 5/78 (6%) sequences. M184IV (66/78, 78%) and K65ENR (29/78,
285 37%) were the most common NRTI mutations detected, whilst K219EQR (23/78, 30%)
286 was the most common thymidine analogue mutation (TAM) detected. The most
287 common NNRTI mutations were K103NS (47/78, 60%) and V106AIMT (27/78, 35%).

288

289 Of the 75 sequence pairs with HIVDR mutations, 18 (23%) had at least one discordant
290 mutation resulting in different phenotypic predictions (Supplementary Table S6). To
291 assess true discordances between sequence pairs, chromatograms were reviewed at
292 each discordant amino acid position by a second laboratory scientist to verify whether
293 the discordances resulted from true mutation calls, or were a result of subjective calling
294 of nucleotide bases. Notably, the majority (13/18) of sequence pair discordances were
295 due to nucleotide mixtures, i.e. positions containing more than one nucleotide, with
296 minor peak height being ≥25% of major peak height. However, only 2 of the discordant
297 sequences were clinically significant, resulting in prediction of a different subsequent
298 ART regimen, as shown in Table 4. Both participants were on efavirenz (EFV)-based
299 first-line ART and had discordances in the NRTI and NNRTI mutations. Example

300 chromatograms of discordances due to mixed bases in the two sequence pairs are
301 shown in Supplementary Figure S2.
302
303

304 **Table 4** Summary of discordant mutations affecting choice of subsequent ART regimen

305

		Protease mutations		Reverse transcriptase mutations		Discordant phenotypic predictions		Mutation score		Predicted regimen of choice	
IDR ID	**Regimen**	**CAP103**	**IDR**	**CAP103**	**IDR**	**CAP103**	**IDR**	**CAP103**	**IDR**	**CAP103**	**IDR**
036	TDF+XTC+EFV	None	None	**M41L**,D67N,	**A62V**,**K65R**,	ABC: H	ABC: H	65	75	TDF+3TC+DTG	AZT+3TC+DTG
				K70R,M184V,	D67N,M184V,	AZT: **H**	AZT: **S**	70	5		
				K219Q	K219Q	FTC: H	FTC: H	70	95		
						3TC: H	3TC: H	70	95		
						TDF: **L**	TDF: **H**	20	65		
				V106M,V179D	V106M,V179D,	DOR: **I**	DOR: **H**	50	100		
					F227FL	EFV: H	EFV: H	90	105		
						ETR: PL	ETR: PL	10	10		
						NVP: H	NVP: H	90	120		
						RPV: L	RPV: L	25	25		
094	TDF+XTC+EFV	None	None	**A62AV**,**K65KR**,	M184V	ABC: **H**	ABC: **L**	70	15	AZT+3TC+DTG	TDF+3TC+DTG
				M184V,**K219KN**		AZT: S	AZT: S	-10	-10		
						FTC: H	FTC: H	95	60		
						3TC: H	3TC: H	95	60		
				K103N,V106M	K103N,V106M	TDF: **H**	TDF: **S**	60	-10		

306 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; DTG, dolutegravir; EFV, efavirenz; FTC, emtricitabine; H, high-level resistance; I, intermediate resistance; L, low-level
307 resistance; PL, potential low-level resistance; TDF, tenofovir; S, susceptible; XTC, lamivudine or emtricitabine

Phylogenetic analysis

Phylogenetic tree reconstruction showed good concordance between sequence pairs of CAP103 and IDR study sequences, and all samples clustered around HIV-1 subtype C (HIV-1C) reference sequences, as shown in Figure 3. Sequences generated from CAP103 study can be identified with a linked KP15 identification number.

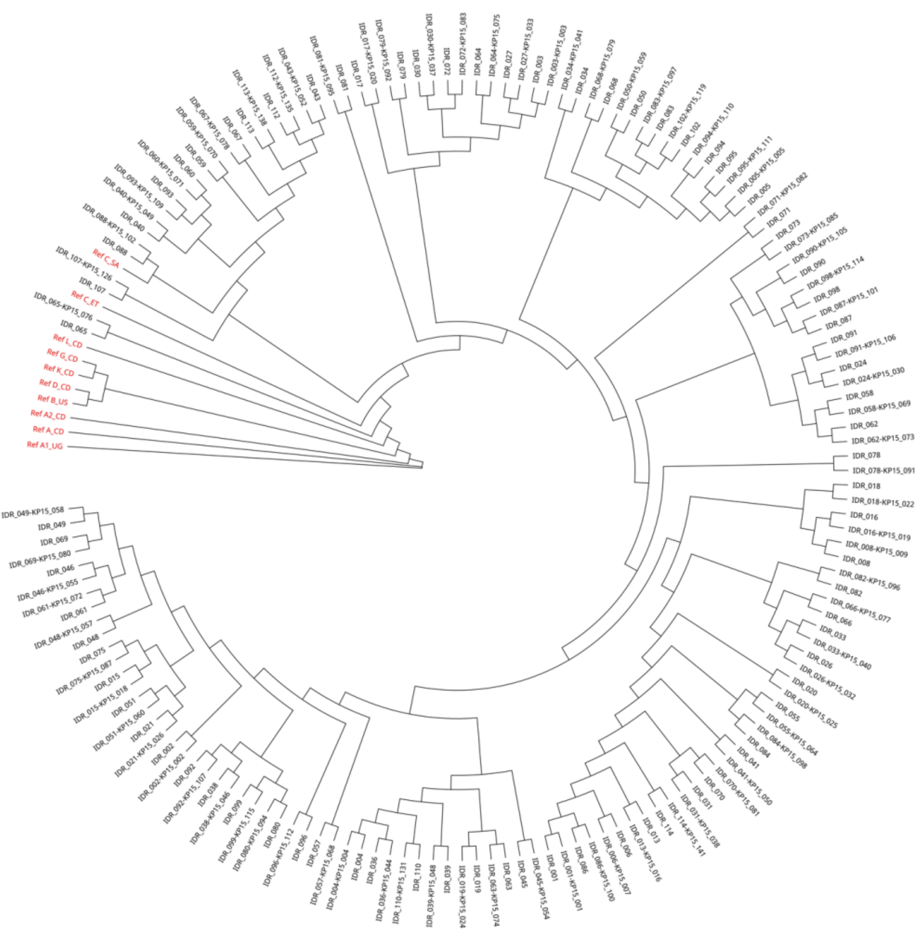


Figure 3 Maximum likelihood phylogenetic tree analysis of IDR and CAP103 sequence pairs

317 **IDR method validation**

318 Of 7 EQA samples, HIVDR mutations from IDR method showed 100% concordance
319 with EQA panel results. Stanford HIVdb and REGA subtyping tool confirmed that
320 sequences were from different HIV-1 subtypes, including subtypes A, C and D. Table
321 5 provides a summary of the EQA samples processed, their HIV-1 subtype
322 classification, and specific mutations detected.

323

324 **Table 5** Summary of EQA control samples processed for IDR method validation

Sample IDs	IDR IDs	Genes compared	HIV-1 subtype	Mutations
11.02A	EQA01	IN	A	**IN:** None
AA05694017	EQA02	PR and RT	C	**PI:** L10F,D30N,N88D **NRTI:** M41L,E44D,D67N,T69D,M184V,L210W,T215Y **NNRTI:** A98G
AA05694021	EQA03	PR and RT	D	**PI:** None **NRTI:** None **NNRTI:** None
AA05694023	EQA04	PR and RT	C	**PI:** M46I,I54V,V82A **NRTI:** M184V **NNRTI:** None
AA05694704	EQA05	PR, RT and IN	C	**PI:** None **NRTI:** D67N,K70R,M184V,K219Q **NNRTI:** None **IN:** None
AA05694734	EQA06	PR, RT and IN	D	**PI:** None **NRTI:** None **NNRTI:** None **IN:** None
AA05694741	EQA07	PR, RT and IN	C	**PI:** L10F,D30N,N88D **NRTI:** M41L,E44D,D67N,T69D,M184V,L210W,T215Y **NNRTI:** A98G **IN:** None

325 EQA, external quality assessment; IN, integrase; NA, not applicable; NRTI, nucleoside reverse transcriptase
326 inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PR, protease; RT,
327 reverse transcriptase

328

329 **Cost-estimate analysis**

330 We estimated the cost of genotyping with the IDR method at ~US$43 per sample. In
331 the case of failure to amplify using the IDR single-fragment approach, we designed an
332 alternative two-fragment approach which we estimated to be ~US$49 (Supplementary
333 Tables S7 and S8). The turnaround time required to genotype all relevant viral *pol*
334 genes using the IDR method was estimated at ~15 hours (~2 working days). Common
335 in-house methods (such as the Applied Biosystems HIV-1 Genotyping kit used for
336 CAP103 genotyping) take approximately ~3 working days to genotype PR and RT
337 genes only (Supplementary Figure S3).

338

339 ## Discussion

340 As more countries in RLS roll-out DTG in first-line regimens as recommended by the
341 WHO [7], the demand for INSTI resistance testing will substantially increase and a
342 population level increase in INSTI resistant mutations is expected [22]. Common
343 methods of HIVDR testing use an approach of genotyping the PR and RT genes
344 separately from the IN gene. This is preferred because of more efficient amplification
345 of shorter gene fragments. However, because of the need to genotype two separate
346 fragments, such an approach doubles the workload and increases cost of genotyping,
347 adding pressure on an already strained health care system. We designed a HIVDR
348 method that effectively counteracts challenges associated with generating separate
349 gene fragments. This simplifies the genotyping process, providing drug resistance
350 profiles for all relevant viral genes at a low cost, with shorter turnaround times. These
351 advantages over current methods of HIV-1 genotyping make the single-assay method
352 ideal for use in RLS.

353

354 In this study we validate our method using 7 blinded remnant EQA samples from
355 QCMD and apply the method to genotype remnant plasma samples from a previous
356 CAPRISA study. QCMD is an international programme that offers quality assessment
357 in molecular diagnostics to ensure laboratory proficiency testing and competence.
358 Assessment of HIVDR genotyping is based on sequence alignment against all
359 participants sequences submitted in the programme. We observed 100%
360 concordance in detection of all drug resistance mutations, demonstrating the IDR
361 method as reliable for HIVDR genotyping of the HIV-1 PI, RT and IN genes. With only
362 3/7 non-subtype C sequences (i.e. one HIV-1 A and two HIV-1D), further assessment
363 of the method to non-subtype C sequences is warranted.

364

365 Using the IDR method, we did not detect any major primary INSTI resistance
366 mutations. Only one of the 78 sequences had an INSTI accessory mutation E157Q.
367 E157Q is a common polymorphic mutation observed in INSTI-naïve patients [31,32].
368 When present alone, E157Q causes only potential low-level resistance to first-
369 generation INSTIs (i.e. elvitegravir and raltegravir), with no resistance to DTG,
370 cabotegravir and bictegravir [29]. However, it causes intermediate resistance to all
371 INSTIs when it occurs together with R263K (a common INSTI mutation at ART failure),
372 whilst decreasing DNA binding activity [33,34]. As expected, we identified similar
373 proportions of PI, NRTI and NNRTI drug resistance mutations as compared to
374 previous CAP103 genotypes, with M46I, M184IV and K103NS being the most
375 common mutations detected, respectively.

376

377 The two discordant sequences (IDR036 and IDR094) resulting in different choice of
378 subsequent ART regimens had the M184V mutation which causes high-level
379 resistance to 3TC and emtricitabine (FTC), whilst increasing viral susceptibility to
380 zidovudine (AZT) and TDF [29]. Sequence IDR036 had multiple RT mutations, with
381 discordances observed in both NRTI and NNRTI mutations. CAP103 detected TAMs
382 M41L and K70R which were not detected in IDR, whilst IDR detected A62V and K65R
383 mutations which were not detected in CAP103. K70R alone causes intermediate
384 resistance to AZT, with M41L playing a minimal role in increasing AZT resistance in
385 the absence of the T215Y mutation [35]. K65R detected by IDR causes high-level
386 resistance to TDF and intermediate resistance to abacavir and 3TC/FTC, even in the
387 absence of other NRTI mutations [29]. Addition of mutations A62V, D67N and K219Q
388 (to K65R) results in high-level resistance to all NRTIs except AZT, thus a predicted
389 AZT-based regimen would be recommended on the basis of the IDR sequence,
390 whereas a predicted TDF-based regimen would be recommended on the basis of the
391 CAP103 sequence. Detection of the mixture F227FL in IDR sequence had no
392 significant impact on other NNRTIs and choice of DTG in subsequent ART regimen.
393

394 Sequence IDR094 had 3 discordant NRTI mutations, all of which were due to detection
395 of nucleotide mixtures in CAP103 sequence. Detection of K65KR alone resulted in
396 high-level TDF resistance thus a preferred AZT+3TC+DTG subsequent regimen for
397 CAP103, as opposed to TLD for IDR. Despite K219KN causing potential low-level
398 resistance to AZT, presence of M184V reduced overall AZT resistance to susceptible.
399 The reason for such discordances is not clear but could be explained by several
400 reasons. Heterogenous distribution of HIV-1 variants in cells (arising from rapid
401 evolution of HIV quasispecies), and absolute number of viral variants obtained during

402 viral RNA extraction at any given time, could result in discordances associated with
403 mixed bases [36]. Other factors to consider include, primer binding preference and
404 location, general sequence quality, and technical errors introduced during PCR from
405 *Taq* polymerase misincorporation [36].

406

407 Amplification and sequencing of larger gene regions by Sanger sequencing is met with
408 challenges in obtaining successful PCR amplification and complete sequence
409 coverage. In this study, we obtained ~91% (87/96) amplification success among
410 samples with VLs ≥1,000 copies/mL (Figure 1). Amplification success was improved
411 by using the two-fragment approach which amplified an additional 9 samples. The 7
412 amplicons with failed sequencing had relatively lower band intensities observed in gel
413 electrophoresis. The two sequences excluded from final analysis (IDR074 and
414 IDR076) showed very high sequence similarity (>98%) after repeating both samples
415 from RNA extraction stage, with no evidence of epidemiological linkage. This
416 suggested potential sample mix-up.

417

418 In efforts to provide genotyping results at the shortest time possible, this method
419 reduces genotyping time from ~3 to ~2 days, saving at least one working day. In
420 addition to providing timely results, it means more samples can be processed over
421 time increasing the capacity of genotypic testing. Also, we deliberately designed the
422 method to use 8 sequencing primers, to make cycle sequencing reaction setup easier
423 for laboratory operators working with standard 96-well plate formats. With this setup,
424 sequencing primers are added in the 8 rows and samples in the 12 columns
425 (Supplementary Figure S4), achieving coverage of all mutations of interest in the HIV-
426 1 *pol* gene.

427

428 With cost remaining one of the major limiting factors to HIVDR genotyping in RLS [37],
429 we estimated the genotyping cost per sample for this method at ~US$43 with an
430 ~US$49 cost for the two-fragment approach as shown in Supplementary Tables S7
431 and S8. These estimates did not include labour and instrument maintenance costs,
432 which tend to vary by region. However, given that common in-house genotyping
433 assays cost between US$48 - US$155 to genotype the PR and RT genes only, with
434 commercial assays ranging between US$155 and US$276 as described previously
435 [37,38], our method provides a cheaper option whilst genotyping not only the PR and
436 RT genes, but also the IN gene.

437

438 There are some limitations to consider. Firstly, the majority of samples processed
439 using the IDR method were HIV-1C samples, the most prevalent subtype accounting
440 for almost half of all HIV infections globally and predominant in RLS [39]. However, we
441 demonstrated successful sequencing of HIV-1A and HIV-1D subtypes in 3 of the 7
442 EQA samples processed. Secondly, prediction of subsequent ART regimens was
443 based on levels of resistance and research evidence, but did not account for other
444 clinical considerations that would typically guide treatment decisions, such as age,
445 weight, co-infections (e.g. Hepatitis B status and tuberculosis), co-morbidities (e.g.
446 renal impairment), and drug contraindications. Thirdly, we mostly compared sequence
447 pairs in the PR and RT genes due to the parent (CAP103) study not having IN gene
448 sequence data, although we would not expect a difference in concordance if paired IN
449 sequences were included. Lastly, use of remnant samples meant that we could not
450 process and compare ~17% of samples (19/115) due to low plasma volumes available

451 for RNA extraction. Also, this potentially affected amplification success rates as RNA
452 tends to degrade with repeated freeze thaw cycles.
453
454 In conclusion, we developed a simple, labour efficient and affordable HIVDR
455 genotyping method for detecting mutations in the HIV-1 PR, RT and IN genes, and
456 demonstrated high concordance with EQA samples. Despite discordances in two
457 sequences resulting in differences in choice of subsequent regimens, recent data from
458 NADIA trial (96-weeks follow up) showed TDF to be superior to AZT when
459 administered with DTG, suggesting both patients would still benefit from switching to
460 TLD. The lower cost, shorter turnaround time, coverage of all genes of interest, and
461 ease of use, make this method ideal and relevant for use in monitoring HIVDR in RLS.
462

463 **List of abbreviations**

464 3TC: lamivudine; ART: antiretroviral therapy; AZT: zidovudine; CAPRISA: Centre for
465 AIDS Programme Research in South Africa; DTG: dolutegravir; EFV: efavirenz; EQA;
466 external quality assessment; FTC: emtricitabine; HIVDR: HIV drug resistance; IN:
467 integrase; INSTI: integrase strand transfer inhibitor; IQR: interquartile range; NHLS:
468 National Health Laboratory Services; NNRTI: non-nucleoside reverse transcriptase
469 inhibitor; NRTI: nucleoside-reverse transcriptase inhibitor; PCR: polymerase chain
470 reaction; PDR; pre-treatment HIV-1 drug resistance; PI: protease inhibitor; PR:
471 protease; QCMD: Quality Centre for Molecular Diagnostics; RLS: resource limited
472 setting; RT: reverse transcriptase; TAM: thymidine analogue mutation; TDF: tenofovir;
473 TLD: tenofovir plus lamivudine and dolutegravir; TLE: tenofovir plus lamivudine and
474 efavirenz; VL: viral load; WHO: World Health Organization

475

476 **Declarations**

477 **Ethics approval and consent to participate**

478 We obtained ethics approval from the Biomedical Research Ethics Committee of the
479 University of KwaZulu-Natal (BREC/000026/2021). Ethics approval was also obtained
480 from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal
481 (BE419/17) for CAP103 study. All participants gave written informed consent to
482 participate in CAP103 study, and also gave written informed consent for sample
483 storage and re-use.
484

485 **Consent for publication**

486 Not applicable.
487

488 **Availability of data and materials**

489 Nucleotide sequence accession numbers for IDR sequences and CAP103 sequences
490 are available from GenBank accession numbers: OM468298 – OM468467 and
491 MW689343 – MW689457, respectively.
492

493 **Competing interests**

494 The authors declare that they have no competing interests.

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501

502 **Authors' contributions**

503 SM, MP, LG, AK, PM, KN and BC conceived, conceptualised, and designed the study.
504 SM, MP and BC performed laboratory experiments. KN and BC prepared original
505 study draft. SM, MP, LG, AK, and PM, reviewed and critiqued the study protocol. SM,
506 MP, LG, AK and BC analysed the data. SM, MP, LG, AK and BC drafted the
507 manuscript, and PM and KN reviewed the manuscript. All authors read and approved
508 the final manuscript.

509

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515

516 **Authors' information**

517 Not applicable

518

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663 **Figure Legends**

664 **Figure 1** Summary flow chart of samples in IDR study from request to analysis

665

666 **Figure 2** Complete sequence coverage of PR, RT and IN genes with 8 sequencing

667 primers

668

669 **Figure 3** Maximum likelihood phylogenetic tree analysis of IDR and CAP103

670 sequence pairs

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682 **Supplementary Material**

683 **Figure S1** Gel image showing representation of amplicons after single-fragment
684 PCR amplification
685
686 **Figure S2** Comparison of discordant mutations between IDR and CAP103
687 sequences due to mixed bases
688
689 **Figure S3** Comparison between IDR method and common in-house HIVDR
690 genotyping workflows
691
692 **Figure S4** Sample and sequencing primer layout in 96-well reaction plate
693
694 **Table S1** PCR primers used to amplify PR, RT and IN genes using a two-fragment
695 approach
696
697 **Table S2** Reverse transcription and first-round PCR conditions for amplifying PR and
698 RT genes with two-fragment approach
699
700 **Table S3** Second-round PCR conditions for amplifying PR and RT genes with two-
701 fragment approach
702
703 **Table S4** One-step RT-PCR conditions for amplifying the IN gene with two-fragment
704 approach
705
706 **Table S5** Summary of all 96 samples processed and sequence outcome

707

708 **Table S6** Details of discordant mutations resulting in discordant phenotypic

709 predictions between IDR and CAP103 sequence pairs

710

711 **Table S7** Approximate cost for genotypic testing using IDR single-fragment

712 approach

713

714 **Table S8** Approximate cost for genotypic testing using IDR two-fragment approach

715

The manuscript in chapter 3 uses a research paper format to describe the designed method for genotyping all relevant HIV-1 *pol* genes (i.e. PR, RT, and IN), that can be used to guide choice of ART in RLS. The following manuscript (in chapter 4) presents detailed step-by-step instructions of the method described in chapter 3. The protocol is divided into five main sections; 1) total RNA extraction, 2) one-step reverse transcription and second round PCR amplification, 3) gel electrophoresis, 4) sequencing, and 5) sequencing analysis and interpretation of sequence data. This protocol also summarizes a list of materials required as well as reagent catalogue numbers.

CHAPTER 4: AFFORDABLE METHOD FOR GENOTYPING HIV-1 REVERSE TRANSCRIPTASE, PROTEASE AND INTEGRASE GENES: AN IN-HOUSE PROTOCOLS

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Author Contribution:

Conceptualization, S.M. and B.C.; investigation, S.M., M.P. and B.C.; resources, B.C.; writing-original draft preparation, S.M., M.P., L.G., A.K., P.M., K.N., and B.C. editing, S.M., M.P., L.G., A.K., P.M., K.N., and B.C. visualization, S.M. and B.C.; supervision, B.C.; project administration, S.M. and B.C.; funding acquisition, K.N. and B.C.

Affordable method for genotyping HIV-1 reverse transcriptase, protease and integrase genes: an in-house protocol

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HIV drug resistance (HIVDR) remains a major threat to achieving sustainable viral suppression on antiretroviral treatment. To overcome concerns over increasing levels in pretreatment drug resistance and subsequent cost implications, most countries including those in resource limited settings (RLS) adopted use of dolutegravir (DTG), a potent integrase strand transfer inhibitor (INSTI). As more people receive INSTIs, the demand for INSTI resistance testing is increasing. Current HIVDR testing methods in RLS focus on genotyping HIV protease (PR) and reverse transcriptase (RT) genes, separate from the integrase (IN) gene. However, such an approach is expensive and evidently increases the workload for HIVDR genotyping. As a result, affordable and labour efficient methods that genotype all relevant HIV-1 genes (i.e., the PR, RT and IN genes) are required to guide clinical decisions, especially in RLS where cost is a major limiting factor. Therefore, we developed a protocol for genotyping complete HIV-1C genes at an affordable cost and time-efficient manner, that can be adopted for use in HIV drug resistance genotyping. The main limitation to this protocol is that it was only tested on HIV-1C samples, and thus might not be effective against other HIV subtypes. In conclusion, the expected results of using this protocol include complete HIV-1C sequence coverage of all relevant viral gene regions; i.e. PR codons 1 - 99, RT codons 1 - 560, and IN codons 1 - 288, for HIVDR genotyping.

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Introduction
This protocol describes a simple, labour efficient and affordable genotyping method for detecting HIVDR mutations in the HIV-1C protease, reverse transcriptase and integrase genes. This method was produced at the Department of Virology National Laboratory Services, University of KwaZulu-Natal, in Durban, South Africa.

Briefly, a simple and innovative single assay polymerase chain reaction (PCR) technique was designed to amplify an approximate 2.9kb HIV-1 pol gene. The method was designed to genotype HIV-1 pol genes with complete coverage of all regions with known HIV drug resistance mutations by overlapping 8 sequencing primers. The amplicons generated are sequenced on an ABI Genetic Analyzer, with HIVDR mutations determined using the Stanford HIV drug resistance database.

Purpose
The purpose of this protocol is to provide detailed instructions that should be followed when genotyping HIV-1 pol genes from plasma samples using BigDye Terminator v3.1 cycle sequencing kit (LifeTechnologies) and Sanger sequencing.

Competing Interests
There are no competing interests to declare.

Data availability
https://www.ncbi.nlm.nih.gov/nuccore/OM468298

LIST OF MATERIALS USED WITH CATALOGUE NUMBERS

Extraction
- NucliSENS easyMAG consumables - 280135 & 280133

PCR
- SuperScript IV One-Step PCR reagents - 12594100
- Platinum Taq reagents - 10966018
- dNTP Mix (10mM each) - R0191

Gel electrophoresis
- TopVision Agarose Tablets - R2801
- UltraPure TBE Buffer, 10X - 15581044
- Orange DNA Loading Dye (6X) - R0631
- SYBR Safe DNA Gel Stain - S33102
- GeneRuler 1 kb DNA Ladder - SM0314

Product Purification
- ExoSAP-IT purification kit - 75001.1.EA

Sequencing
- BigDye Terminator v3.1 Cycle Sequencing kit - 4337455
- BigDye XTerminator Purification kit - 4376487

Consumables
- Tips
- MicroAmp 8-Tube Strip - A30589
- Adhesive PCR Plate Foils (nuclease-free) - AB0626
- MicroAmp Optical 96-Well Reaction Plate - N8010560
- Nuclease-free Water - AM9932
- 1.5ml nuclease free eppendorf tubes - AM12400
- Personal protective equipment

Instruments and software
- Pipettes
- Refrigerated microcentrifuge
- Biosafety cabinets
- Vortexer
- NucliSENS easyMAG
- Mini-centrifuge
- Thermal cycler
- Gel electrophoresis unit
- UV detection unit
- Plate centrifuge
- Genetic Analyzer
- Geneious software
- Stanford HIV drug resistance database

The use of potentially infectious material requires that all persons must use laboratory safety protective clothing. It is advisable for all users to have undergone safety training to ensure safe handling and disposal of harmful material. The disinfection of work areas is required before and after work.

- Ensure that the working areas are disinfected
- Remember to check the expiring date of the reagents before use
- Ensure that instruments maintenance is up to date

EXTRACTION

1 **Sample preparation and RNA extraction on NucliSENS easyMAG**

1.1 Retrieve plasma samples from **-80 °C** freezer and leave to equilibrate to room temperature prior to processing.

1.2 Transfer **500 µL** of plasma into well labeled **1.5 mL** Sarstedt screw-cap tubes/ eppendorf tubes and place in a pre-cooled microcentrifuge at **4 °C** .

1.3 Mark the top of each tube on the outside and spin at **23000 x g** for **01:00:00** at **4 °C** .

1h

1.4 After spinning, remove **300 µL** of supernatant and briefly vortex the ~ **200 µL** pellet.

Preferably remove ~ **280 µL** to ensure you have at least **200 µL** of pellet remaining. Pipette from the opposite side of the mark on the top of the tube.

1.5 Proceed to extraction room

Prior to extraction, make **400 µL** aliquots of lysis buffer and remove magnetic silica from fridge and equilibrate to room temperature.

1.6 Pipette mix the 200 µL pellet and transfer to 400 µL of lysis buffer.

1.7 Vortex briefly and incubate at room temperature for 00:20:00 .

20m

1.8 While the sample is lysing, label disposable NucliSENS easyMAG consumables according to sample list.

1.9 After incubation, transfer lysate (~ 600 µL) into respective wells of NucliSENS easyMAG consumables.

1.10 Vortex magnetic silica briefly and add 50 µL to numbered NucliSENS easyMAG consumables.

1.11 Using a BioHit multichannel pipette set at P3, mix sample solution with magnetic silica by pipetting up and down, avoiding bubbles. Remove excess bubbles if necessary.

1.12 Load NucliSENS easyMAG consumables on to the NucliSENS easyMAG instrument and scan barcodes on consumables.

1.13 Program NucliSENS easyMAG instrument and select 25 µL elution volume.

1.14 Start extraction run.

> The run will take ~ 00:35:00

1.15 After extraction is complete, transfer eluent into appropriately labelled 1.5 mL microcentrifuge tubes.

Discard NucliSENSeasyMAG consumables appropriately as biohazard waste.

1.16

1.17 Proceed to perform PCR amplification or store RNA at -80 °C until use.

PCR MASTER MIX

2 Preparation of one-step reverse transcription and second-round PCR master mix

2.1 Proceed to PCR master mix room (i.e. clean room).

2.2 All reagents except the enzymes (Superscript IV and Platinum *Taq* DNA Polymerase) must be thawed and vortexed for 00:00:05 to 00:00:10 15s

2.3 Label reverse transcription and second-round PCR tubes 0.2 mL with sample numbers, accordingly.

2.4 Label two 1.5 mL microcentrifuge tubes as follows; Tube 1: for RT one-step PCR master mix, and Tube 2: for second-round PCR master mix.

> Prepare each master mix for the number of specimens to be amplified, including a negative control, and positive control.

> Add reagents in order listed in Table 1, and make two extra volumes to account for pipetting errors.

2.5 Prepare RT-PCR master mix as shown in Table 1, using Superscript IV one-step PCR reagents.

A	B	C
Reagents	**Volume per reaction (µl)**	**Concentration per reaction**
2X Reaction RT-PCR master mix	12.5	1X
Nuclease-free Water	2.25	-
PANA2AF (5µM)	2.5	0.5µM
PANA3AR (5µM)	2.5	0.5µM
SSIV/ Platinum SuperFi DNA polymerase (2X)	0.25	0.02X
Total volume	**20**	-

Table 1. One-step reverse transcription PCR master mix.

Details of primers for one-step RT-PCR master mix are shown in Table 2.

A	B	C
Primer name (Direction)	**Primer sequence**	**HXB2**
PANA2AF (Forward)	GAGGCAATGAGCCAARCAAACA	1882 - 1903
PANA3AR (Reverse)	TTCCAGGGCTCTAGKTTAGG	5846 - 5865

Table 2. Details of primers used in one-step reverse transcription PCR master mix.

Add reagents in order listed in Table 3, and make two extra volumes to account for pipetting errors.

2.6

Prepare master mix for the second-round PCR as shown in Table 3, using Platinum *Taq* PCR reagents.

A	B	C
Second-round PCR Mastermix		
Reagent	**Volume per reaction (µl)**	**Concentration per reaction**
Nuclease-free Water	18.4	-
10X PCR Buffer	2.5	1X
MgCl2 (50mM)	1.0	2mM
dNTP (10mM)	0.5	0.2mM
Pro1 (5µM)	0.25	0.05µM
5066R (5µM)	0.25	0.05µM
Platinum *Taq* DNA Polymerase	0.1	-
Total volume	**23**	-

Table 3. Second-round PCR master mix.

Details of primers for second-round PCR master mix are shown in Table 4.

A	B	C
Primer name (Direction)	**Primer sequences**	**HXB2**
Pro1 (Forward)	TAGAGCCAACAGCCCCACCA	2147 - 2166
5066R (Reverse)	ATCATCACCTGCCATCTGTTTTCCAT	5041 - 5066

Table 4. Details of primers used in second-round PCR.

2.7 Mix one-step RT-PCR master mix by pipetting up and down (or by gently tapping the tube), and transfer **20 µL** to the respective **0.2 µL** PCR tubes.

2.8 Mix second-round PCR master mix by pipetting up and down (or by gently tapping the tube), and transfer **23 µL** to the respective **0.2 µL** PCR tubes.

2.9 Take aliquoted one-step RT-PCR master mix into the extraction laboratory (or dead air space cabinet in general laboratory).

> Store second-round PCR master mix at **-20 °C** until use.

ADDING RNA

3 Adding RNA to one-step RT-PCR master mix.

3.1 Proceed to extraction laboratory, or dead air space cabinet.

3.2 Thaw RNA, gently tap tubes, and briefly spin.

3.3 Add **5 µL** RNA to respective tubes containing master mix.

> Add **5 µL** of nuclease free water to negative control. Total reaction volume is **25 µL** .

3.4 Proceed to the PCR amplification room.

ONE-STEP RT-PCR

4 RT-PCR amplification process.

4.1 Gently tap PCR tubes to mix and centrifuge briefly.

4.2 Switch on thermal cycler and wait for the instrument to initialize.

4.3 Place tubes in thermal cycler and run the conditions shown in Table 5.

A	B	C	D
Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
cDNA synthesis	50	10 minutes	1
Pre-denaturation	98	2 minutes	1
Denaturation	98	10 seconds	40
Annealing	56	20 seconds	
Extension	72	2 minutes	
Final extension	72	10 minutes	
Hold	4	∞	Hold

Table 5. One-step RT-PCR amplification conditions.

PCR run takes ~ **02:35:00**

4.4 After PCR is complete, remove PCR tubes from thermal cycler and proceed to second-round PCR amplification, or store at **-20 °C** .

SECOND-ROUND PCR

5 Second-round PCR amplification.

5.1 In PCR amplification laboratory, remove second-round PCR master mix from **-20 °C** .

5.2 Gently mix one-step RT-PCR amplicons by pipetting up and down, and add **2 µL** to the respective second-round PCR master mix tubes.

Total reaction volume is **25 µL** .

5.3 Gently tap PCR tubes and centrifuge briefly.

5.4 Place the PCR tubes in thermal cycler and run the conditions shown in Table 6.

A	B	C	D
Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
Pre-denaturation	94	2 minutes	1
Denaturation	95	10 seconds	40
Annealing	56	20 seconds	
Extension	72	2 minutes	
Final extension	72	10 minutes	1
Hold	4	∞	Hold

Table 6. Second-round PCR amplification conditions.

PCR run takes ~ **02:15:00** .

5.5 After PCR is complete, remove PCR tubes from thermal cycler and proceed to run gel electrophoresis, or store second-round amplicons at **-20 °C** .

DETECTION OF AMPLICONS

6 Gel electrophoresis on agarose gel.

6.1 Assemble gel casting apparatus by securing gel casting gates on both sides of the casting tray. Place gel comb in upper slot of the casting tray.

6.2 Prepare 1X TBE by adding 1 part 10X TBE buffer to 9 parts distilled water. For example to make **1 L** of 1X TBE, mix **100 mL** of 10X TBE with **900 mL** of distilled water.

6.3 Prepare 1% agarose gel by adding two **0.5 g** agarose tablets to **100 mL** of 1X TBE in a Schott bottle.

6.4 Heat in microwave for approximately 00:02:00 , swirling occasionally to ensure agarose tablets have dissolved completely.

2m

> Take care that the gel-mix does not boil over.

6.5 Gently remove from microwave taking care not to spill over and allow cooling at room temperature, or by running the base of the flask under cold water.

> The gel is cool enough when you are able to comfortably touch the gel bottle for **00:00:03** to **00:00:05** .

6.6 Add **10 µL** of SYBR safe gel stain (10,000X concentrate) to **100 mL** gel.

> Add **1 µL** of SYBR safe gel stain (10,000X concentrate) for every **10 mL**) of agarose gel mix.

6.7 Allow agarose gel to set (~ **00:30:00**).

30m

6.8 Once set, carefully remove gel comb and the casting gates.

6.9 Place agarose gel in the electrophoresis tank with wells closest to the anode (i.e. negative electrode (black) position on top) and immerse in 1X TBE buffer.

6.10 Add **1 µL** of gel loading dye to a piece of parafilm paper for each amplicon to be loaded on the gel.

6.11 Mix with 5 µL of each amplicon and load into respective wells of the agarose gel.

> Preferably, start loading amplicons from the second well, taking note of the order by which the amplicons are loaded.

6.12 Mix 3 µL of gel loading dye with 3 µL of DNA ladder, and load into the first well of the agarose gel.

6.13 Close the lid of the electrophoresis tank ensuring that the negative electrode (black) is positioned close to the agarose gel wells.

6.14 Switch PowerPac on and set voltage at 120V.

6.15 Run the gel for at least 00:45:00 .

45m

> Check for bubbles at negative electrode (black) to ensure there is current running through the tank.

6.16 After running gel, remove it from electrophoresis tank and visualize on BioMetra UV detection unit.

6.17 PCR amplicons should be at a size of approximately 2.9 kilobases (kb) as measured against the DNA ladder in well position 1.

> For a valid PCR run, the positive control should have a band at approximately 2.9kb, and the negative control should not have a band.

6.18 Proceed to PCR product purification or store amplicons at -20 °C until use.

PCR PRODUCT PURIFICATION

7 PCR product purification using ExoSAP-IT PCR production purification

7.1 For samples with successful amplification, label PCR tubes required for purification.

7.2 Tap amplicon tubes gently to mix and briefly spin to bring the contents to bottom of tube.

> If frozen, first thaw the amplicons to room temperature.

7.3 Remove ExoSAP-IT Express reagent from -20 °C freezer.

7.4 Gently tap ExoSAP-IT Express reagent to mix and briefly spin to bring contents to bottom of tube.

7.5 Mix **10 µL** of PCR amplicon with **4 µL** of ExoSAP-IT Express reagent for a combined **14 µL** reaction volume.

7.6 Mix thoroughly by tapping the tube and quick spin to bring contents to the bottom of the tube.

7.7 Place the tubes in thermal cycler and run the conditions shown in Table 7.

A	B	C	D
Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
Cleanup	37	4 minutes	1
Enzyme inactivation	80	1 minute	1
Hold	4	∞	Hold

Table 7. PCR purification conditions.

7.8 After ~ **00:05:00** on thermal cycler, purified PCR products are ready for DNA sequencing. 5m

Purified PCR products may be stored at **-20 °C** until use.

CYCLE SEQUENCING REACTION AND PURIFICATION

8 **BigDye cycle sequencing.**

8.1 The following procedure is used to set up the sequencing reaction mix using BigDye Terminator v3.1 cycle sequencing kit (LifeTechnologies) with 8 primers per sample.

8.2 Thaw all reagents at room temperature.

8.3 Label eight **1.5 mL** tubes, one for each primer mix.

8.4 Vortex sequencing buffer and primers and briefly spin to bring contents to the bottom of the tube.

8.5 Mix BigDye reagent by tapping tube and briefly spin to bring contents to bottom of tube.

Add reagents in order listed in Table 8, and make two extra volumes to account for pipetting errors.

8.6 Prepare master mix for each primer as shown in Table 8.

A	B	C
Reagent	**Volume (µl)/ reaction**	**Concentration per reaction**
Nuclease-free Water	6.1	-
5X Sequencing buffer	2	1X
Primer (3.2µM)	0.5	0.2µM
BigDye Sequencing mix	0.4	-
Total	**9.0**	

Table 8. BigDye cycle sequencing reaction mix.

Details of sequencing primers are shown in Table 9.

A	B	C	D
Primer (Direction)	**Primer sequence**	**HXB2**	**Gene**
RTC1F (Forward)	ACCTACACCTGTCAACATAATTG	2486 - 2508	PR and RT
RTC2R (Reverse)	TGTCAATGGCCATTGTTTAACCTTTGG	2630 - 2604	PR and RT
RTC3F (Forward)	CACCAGGGATTAGATATCAATATAATGTGC	2965 - 2994	PR and RT
RTC4R (Reverse)	CTAAATCAGATCCTACATACAAGTCATCC	3101 - 3129	PR and RT
KVL076 (Forward)	GCACAYAAAGGRATTGGAGGAAATGAAC	4161 - 4188	IN
KVL082 (Forward)	GGVATTCCCTACAATCCCCAAAG	4647 - 4669	IN
KVL083 (Reverse)	GAATACTGCCATTTGTACTGCTG	4750 - 4772	IN
PAN2R (Reverse)	CTGCCATCTGTTTTCCATAYTC	5037 - 5058	IN

Table 9. Details of sequencing primers.

8.7 Label 96-well plate for the cycle sequencing reaction PCR.

8.8 Dispense **9 µL** of each primer mix into the respective wells of a 96-well plate.

8.9 Add **1 µL** purified amplicons to the respective wells.

> It is recommended to add the eight primers across the plate (e.g. primer RTC1F in wells A1 - A12), with samples added in plate columns (e.g. sample 1 in wells A1 - H1).

8.10 Cover plate with aluminium plate seal, tap plate gently to mix, and spin plate briefly.

8.11 Remove aluminium plate seal and cover plate with PCR septa mat.

8.12 Place on thermal cycler and run the conditions shown in Table 10.

A	B	C	D
Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
Pre-denaturation	96	1 minutes	1
Denaturation	96	10 seconds	35
Annealing	50	5 seconds	
Extension	60	4 minutes	
Hold	4	∞	

Table 10. Cycle sequencing reaction conditions.

8.13 After the cycle sequencing reaction run is complete, proceed with BigDye sequencing reaction purification, or store the plate covered in foil at **4 °C** for up to 1 week, or at **-20 °C** for storage longer than 1 week.

8.14 Remove SAM and XTerminator reagents from fridge (stored at 4 °C) and equilibrate to room temperature prior to preparing SAM/BigDye XTerminator mix.

> The BigDye XTerminator Purification kit (Life Technologies) is used to purify sequencing reaction products. The kit consists of 2 reagents; XTerminator solution and SAM solution.

8.15 Following BigDye cycle sequencing reaction, centrifuge 96-well reaction plate at 4000 rpm for 00:01:00 . 1m

8.16 Thoroughly vortex XTerminator solution for at least 00:00:30 . 30s

8.17 Prepare the SAM/BigDye XTerminator bead working solution in a 50 mL Falcon tube, by adding reagent volumes shown in Table 11.

A	B
Component	**Volume per 10µL reaction**
SAM solution	45µL
BigDye XTerminator solution	10µL
Total volume	**55µL**

> Preferably use wide-orifice tips when pipetting BigDye XTerminator solution.

8.18 Mix the SAM/BigDye XTerminator bead working solution thoroughly by vortexing and dispense in a reagent reservoir.

8.19 Remove PCR septa mat from 96-well reaction plate and add 55 µL of SAM/BigDye XTerminator bead working solution to each well.

> Preferably use a multi-channel pipette to add reagent mix, changing tips

each time.

8.20 Cover plate with aluminium plate seal.

8.21 Vortex plate on a plate shaker at **1800 rpm** for **00:30:00** . 30m

8.22 After vortexing, centrifuge plate at **4000 rpm** for **00:02:00** . 2m

If the reaction plates are not run immediately, you can store them under the following conditions:

- Room temperature (i.e. **20 °C** - **25 °C**): Store plates sealed with adhesive film or septa for up to 2 days.
- Refrigerated storage (**4 °C**): Store plates sealed with adhesive film for up to 10 days.
- Frozen storage (**-20 °C**): Store plates sealed with adhesive film for up to 10 days.

CAPILLARY ELECTROPHORESIS

9 Capillary electrophoresis on 3730 Genetic Analyzer

9.1 Setup plate run on Genetic Analyzer (Applied Biosystems), according to cycle sequencing reaction plate layout.

9.2 Load plate onto Genetic Analyzer (Applied Biosystems) and start capillary electrophoresis run.

9.3 At the end of the run, remove the plate from the Genetic Analyzer and dispose in biohazard waste.

9.4 Login to SeqA6 software on a computer connected to the Genetic Analyzer

and run primary analysis.

9.5 Save the analyzed data files, and copy the ab1 sequence files to USB disk.

SEQUENCE ANALYSIS

10 Sequence editing and drug resistance interpretation.

10.1 Upload ab1 sequence files from USB key into Geneious software (Biomatters Ltd, New Zealand).

10.2 Trim and edit sequences for good quality reads.

> If you are in the correct reading frame, the beginning of protease gene should start with the amino acids PQITLW, the beginning of reverse transcriptase gene should start with PISPIE, and the beginning of integrase gene should start with FLDGID.

10.3 Align all sequence files for each sample to an annotated HIV reference sequence, and edit sequences where necessary. This entails identifying any ambiguities, and verifying positions with mixed bases by inspecting quality (symmetry, height, and background) of the base calls.

10.4 Align all consensus sequences and perform phylogenetic tree reconstruction to assess sequence similarity.

> If any two sequences have >97% similarity, all the stages of the protocol should be reviewed, starting with sequence analysis and going back to RNA extraction, to ensure that there was no sample mix up (i.e. sample switching, mislabeling), or contamination.

10.5 Export consensus sequences in FASTA format and assess HIV drug resistance mutations using the Stanford University HIV drug resistance database (https://hivdb.stanford.edu/hivdb/by-sequences/).

CHAPTER 5: SYNTHESIS

5.1 Discussion

DTG has been introduced on a large scale as part of fixed-dose combinations of TDF and 3TC (i.e., TLD) to effectively curb the spread of antiretroviral resistance. It is well tolerated and affordable making it ideal for use in RLS [107]. Based on findings from the NADIA and ARTIST trials, increased distribution of TLD could minimize the need for pretreatment and acquired HIVDR testing in RLS, as individuals on DTG infrequently develop virological failure with clinically significant drug resistance mutations [20,21]. However, surveillance for HIV drug resistance remains critical. For instance, the ADVANCE study showed less than adequate viral suppression rates among individuals on DTG-based ART at 48-weeks, raising concerns over long-term treatment outcomes in a setting with high NNRTI-PDR [108]. Thus, genotypic drug resistance testing could facilitate the choice of ART regimens in most settings where DTG has been rolled-out and enable clinicians to determine which individuals with virological failure on DTG-based ART require a change in treatment.

HIVDR genotyping can assist in the selection of optimal ART regimens which will help to attain the third 95-95-95 UNAIDS target for sustainable VL suppression. However, limited laboratory capacity and high operational costs remain major limiting factors to routine drug resistance testing in many RLS. Genotypic resistance testing using inexpensive PMAs would be useful in RLS where resources, capacity, and infrastructure to perform standard genotypic drug resistance testing are limited [109]. However, the disadvantages of PMAs evolve around requirement for multiple tests per target site which increases the costs of PMAs, especially without affordable multiplexing assays [89]. This suggests that in the short-term in-house Sanger-based genotypic resistance testing will remain the predominant technology, and NGS has great potential for the medium-to-long term, offering the possibility of multiplexing very large numbers of samples in a single sequencing reaction, and lowering costs even further in high-throughput laboratories. The review paper on chapter 2 discussed use of Sanger sequencing and NGS methods for HIVDR genotyping, focusing on their use in RLS. The paper also showed several HIVDR assays, none of which detect mutations in PI, RT and IN genes concurrently. Furthermore, the paper discussed knowledge gaps and offered recommendations on how to overcome existing barriers for implementing HIVDR genotyping in RLS, highlighting a need to design Sanger sequencing assays that detect all relevant mutations in a single amplicon, in order to simplify HIVDR genotyping for clinical diagnostics and research use.

As previously mentioned, common methods of HIVDR testing use an approach of genotyping the PR and RT genes separately from the IN gene. This is preferred because of simpler amplification of shorter gene fragments. However, because of the need to genotype two separate gene fragments, such an approach doubles the workload and increases cost of genotyping, adding pressure on an already strained healthcare system. Genotyping all relevant HIVDR mutations using a quick and affordable method is important for guiding treatment options and patient management in RLS. The method designed and reported in this thesis, provides a positive step towards effectively counteracting challenges associated with HIVDR testing, whilst using similar techniques already in place. This approach simplifies the genotyping process, providing drug resistance profiles for all relevant viral genes at a low cost and with shorter turn-around times, making it ideal for use in RLS.

In-house methods are more flexible than kit-based methods, considering how changes to primers can be more easily implemented when required [110]. Chapters 3 and 4 describe a typical in-house assay developed using innovative techniques, whereby specific PCR and sequencing primers are adapted for HIVDR genotyping. Despite sequence pair analysis of PR and RT genes showing differences between the designed in-house method (hereafter referred to as IDR method) and CAP103, there was a 100% concordance in identifying samples with at least one HIVDR mutation, similar to a previous method reported by Manasa et al. (100%) [28]. Average nucleotide and amino acid pairwise identities were >99%, demonstrating high sequence quality, with the WHO recommending a minimum of 90% sequence similarity for genotyping assays [110]. Similarly our results were in agreement with those achieved by Zhou et al, who reported 99.4% nucleotide identity [111]. Despite the overall sequence similarities, we observed some mutation discordances in sequence pairs.

Chapter 3 shows that about 22% (17/79) of sequence pairs had a discordant mutation resulting in different phenotypic predictions, although it affected the predicted choice of subsequent ART regimens in only 2 of the 17 sequences. To assess true discordances between IDR and CAP103 sequences, chromatograms were reviewed at each discordant amino acid position by a second laboratory scientist to verify whether the discordances resulted from true mutation calls, or were a result of subjective calling of nucleotide bases. Of 17 sequence pairs with discordances, approximately 2 of every 3 discordances were due to mixed bases at drug resistance mutation sites. Figure 10 shows examples of discordances due to mixed bases, in sequences IDR036 and IDR094. In the first example (i.e., IDR036), the IDR sequence detected A62AV, K65KR and D67DN as mixtures that were not detected in CAP103

sequence. In the second example (i.e., IDR094), CAP103 sequence detected A62AV and K65KR mutations as mixtures that were not detected in the IDR sequence.

Figure 10 Comparison of discordant mutation positions between IDR and CAP103 sequences

The reason for such discordances is not clear but could be explained by several reasons. Heterogenous distribution of HIV-1 variants in cells (arising from rapid evolution of HIV quasispecies), and absolute number of viral variants obtained during viral RNA extraction at any given time, could result in discordances associated with mixed bases [112]. Other factors to consider include; primer binding preference and location, base calling criteria, general sequence quality, and technical errors introduced during PCR from Taq polymerase misincorporation [112].

Maximum-likelihood phylogenetic analysis (shown in manuscript 3, Figure 3) showed good sequence pair comparison between IDR and CAP103 sequences, with clustering of unique sequence pairs on the same operational taxonomic units, as well as clustering with HIV-1 Subtype C reference sequences. Two sequence pairs (i.e., IDR074 and IDR076) clustered together with high sequence similarity (>98%), suggesting potential sample mix-up. Without adequate plasma to repeat extraction, processing of these samples was repeated from PCR through sequencing, and still showed the same level of similarity. The two samples were excluded from final analysis. A possible explanation would be that

contamination occurred when aliquoting plasma samples for extraction. This can be avoided by being more vigilant at each stage of sample processing, and adhering to crucial good clinical laboratory practices [113]. This shows how phylogenetics can be used as a quality control measure in HIVDR genotyping.

Cost of infrastructure and resources is arguably the greatest limiting factor to HIVDR genotyping access in RLS. In cost-estimate analysis, the running cost per sample for the IDR method was estimated at ~US$43 for a single genotyping attempt, with the cost increasing to ~US$64 after adding cost that accounts for a more than generous 50% genotyping failure rate (Appendix 4, S6 Table). Additionally, the estimated cost per sample for using an alternative two-fragment approach was ~US$49 and increased to about US$73, after accounting for 50% genotyping failure rate (Appendix 4, S7 Table). These estimates do not include labour and instrument maintenance costs. Regardless, IDR method cost estimates are similar to common in-house genotyping assays that cost between US$48 - US$155 to genotype the PR and RT genes only, with commercial assays ranging between US$155 and US$276 [88,89]. Moreover, the IDR method has an added advantage in that it provides genotyping of not only the PR and RT genes, but also the IN gene, at similar costs. Table 3 shows a comparative cost analysis of common in-house methods (with specified cost) in RLS.

Table 3. Comparative cost analysis for in-house HIVDR methods in RLS

Year	Available methods	Country	Sample type	Genes	Cost (US$)
2007	Chen JHK et al. [114]	China	Plasma	PR and RT	$30
2010	Saravanan et al. [115]	India	Plasma	PR and RT	$100
2011	Zhou Z et al. [111]	USA	DBS/ Plasma	PR and RT	$40
2013	Inzaule S et al. [13]	Kenya	DBS	PR and RT	$110
2013	Inzaule S et al. [13]	Kenya	Plasma	PR and RT	$113
2014	Acharya A et al. [116]	India	Plasma	PR and RT	$85
2014	Chaturbhuj DN et al. [117]	India	Plasma	PR and RT	$160
2019	Seatla KK et al. [118]	Botswana	Plasma	IN	$32
2019	Magomere EO et at. [119]	Kenya	Plasma	PR and RT	$59
2022	Manyana S (unpublished)	South Africa	Plasma	PR, RT and IN	$64

DBS, dried blood spots; IN, integrase gene; PR, protease gene; RT, reverse transcriptase gene; US$, United State dollars

In addition to cost, turnaround time is an important factor in making timely treatment decisions. The designed method presented in this thesis is widely implementable for HIVDR genotyping in most RLS laboratories, with a total workflow of ~15 hours from extraction to results. This method results in reduced genotyping time from ~21 hours to ~15 hours, saving at least one working day when compared to other common methods. In addition to providing timely results, it means more samples can be processed over time increasing the capacity of genotypic testing. Also, we deliberately designed the method to use 8 sequencing primers, to make cycle sequencing reaction setup easier for laboratory operators working with standard 96-well plate formats. With this setup, sequencing primers are added in the 8 rows and samples in the 12 columns as shown in Appendix 4 (S2 Figure), achieving coverage of all mutations of interest in the *pol* gene. The simplicity, reduced turnaround time, lower cost and sensitivity in detecting resistance mutations makes this method ideal for use in RLS.

5.2 Recommendations for future research and policy

This thesis presents an ideal HIVDR method for use in RLS. However, the method was only compared to paired PR and RT gene sequences. This may raise concerns around its effectiveness in detecting IN gene mutations. Therefore, future studies similar to this one should consider also comparing paired IN gene sequences. Moreover, all samples used to design the IDR method were from HIV-1C infected individuals, the most common subtype in RLS. However, future research work should consider testing similar in-house methods to non-HIV-1 subtype C samples, including CRFs, and if necessary, subtype-specific primers can be designed to amplify other HIV-1 subtypes. All samples processed had VLs >1000 copies/mL, aligning with the South African HIV treatment guidelines for HIVDR testing in individuals with persistent viremia [110]. Thus, this method might not be as effective in genotyping HIV from samples with VLs <1,000 copies/mL. Assessing sensitivity in detecting low-level viremia is important in future research, as such patients could potentially transmit resistant virus. In addition, future work should also consider HIVDR genotyping from other sample isolates such as DBS, given the advantages of this type of sample over plasma, in terms of transportation and storage.

Future research should also consider focusing on incorporating NGS technologies in routine HIVDR testing for clinical diagnostics. Currently only the Sentosa SQ HIV platform (Vela Diagnostic, Germany) has authorised marketing approval from the US-FDA for use in HIVDR testing. However, the per sample cost of using Sentosa SQ HIV platform (i.e., ~US$400) currently remains a prohibitive factor to use in RLS. In addition, the lack of understanding around the impact of LA-DRVs would have

to be addressed in order to effectively use such NGS technologies in making clinical decisions. Furthermore, future research should focus on reducing variability in interpretation of HIVDR genotyping data. This could be established through development of standard data analysis pipelines/ software to ensure consistency in data interpretation [120]. Ultimately, future research work should aim to design HIVDR methods that are simple, affordable, offer point-of-care testing, standard data interpretation, and are sensitive at detecting HIVDR mutations in a broad range of HIV subtypes and even at low VL thresholds. Integrating such methods into the HIV treatment cascade will help improve treatment decisions and the quality of lives of people living with HIV.

With recent transitions towards use of TLD, countries (especially in RLS) should prioritise sustainable treatment monitoring and reduce time taken to make decisions for switching ART regimens, in order to meet the UNAIDS 95-95-95 targets. Significant progress has been made in South Africa in achieving these targets, especially in diagnosing HIV infected individuals. However, policy makers should enforce measures that improve the number of people that are initiated and remain on ART, considering that only ~69% of people diagnosed with HIV were receiving ART in South Africa by end of 2020 (Table 1) [7]. Moreover, there is also room for improvement in achieving the third 95% UNAIDS target in South Africa, given that viral suppression rates were at ~88% by end of 2020 (Table 1) [7]. Therefore, policy should strengthen timely action on viral load data, repeat viral load testing, and timely switching of ART regimens in accordance with global and local HIV treatment guidelines. Given that TLD is used for first- and second-line regimens, policies should consider resistance testing after failing DTG-based ART, to assess virologic failure due to HIVDR. Furthermore, policies that support structured and continuous HIVDR surveillance using aggregated data from individual patients and population-based surveys, are required to monitor community drug resistance, to improve ART programmes and adapt to changes in ART guidelines, especially in RLS where routine drug resistance testing is unavailable.

5.4 Conclusion

Overall, this research work shows an innovative method for HIVDR genotyping that is simple, timely, and affordable to detect mutations in the HIV-1 PR, RT and IN genes. Similar methods that are sensitive to non-HIV-1C subtypes are required in future to genotype a broader range of HIV subtypes. Furthermore, given this method's cost-estimate and shorter turnaround time, it can be implemented and performed seamlessly in RLS, whilst accommodating changes in ART regimens. In conclusion, low cost HIVDR genotyping methods that are easy to implement and produce results relevant to treatment regimens are required to address the problem of HIVDR. Implementation of these methods, vigilant VL

monitoring, and prompt action on diagnostic results will help improve rates of viral suppression, which will subsequently improve the quality of life of people living with HIV and ultimately help achieve HIV elimination.

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APPENDICES

Appendix 1 Biomedical research ethics approval

05 June 2021

Mr Sontaga Cris Manyana (221116823)
School of Lab Med & Medical Sc
Medical School

Dear Mr Manyana,

Protocol reference number: BREC/00002635/2021
Project title: Innovative and affordable HIV-1 drug resistance testing for resource limiting settings
Degree: MMedSc

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 05 June 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_2_Guidelines_1.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 05 June 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 July 2021.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

Appendix 2 CAP 103 approval letter of support to use remnant samples

Generating Knowledge - Impacting Health

Doris Duke Medical Research Institute (2nd floor), 719 Umbilo Road, Private Bag X7, Congella, 4013, Durban, South Africa
tel: +27 31 2604555 | **fax**: +27 31 2604549 | email: **caprisa@caprisa.org** | **www.caprisa.org**

27 May 2021

Dear Members of the Biomedical Research Ethics Committee

RE: Letter of support for use of remnant samples for a project entitled: Innovative and affordable HIV-1 drug resistance testing for resource limited settings (Provisional BREC number: BREC/00002635/2021)

This letter serves to confirm that the project entitled, "Innovative and affordable HIV-1 drug resistance testing for resource limited settings", has received permission from the Centre for the AIDS Programme of South Africa (CAPRISA), to use remnant plasma samples from the CAPRISA 103 study entitled, "Acquired HIVDR and virologic monitoring in a HIV hyper- endemic setting, in KwaZulu-Natal".

The new project aims to design a drug resistance genotypic testing assay for HIV-1 viral genes relevant to our setting. At CAPRISA we have a rich repository of samples that have been obtained through several research projects. We have full ethical approval from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, to use remnant samples from the CAPRISA 103 study (BE419/17). Please see attached supporting documentation showing the current ethics approval certificate and the consent form that was approved for specimen storage for possible future research.

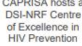
Please do not hesitate to contact me should you have any further questions.

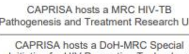
Yours sincerely

Professor Kogie Naidoo

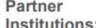
Deputy Director

CAPRISA





Partner Institutions:



Appendix 3 CAP 103 study ethics approval from Biomedical Research Ethics Committee

03 May 2021

Dr Kogieleum Naidoo
CAPRISA
Kogie.naidoo@caprisa.org

Dear Dr Naidoo

PROTOCOL: Acquired HIV drug resistance and virologic monitoring in a HIV hyper endemic setting, in KwaZulu-Natal Province, South Africa. Non-degree
BREC reference number: BE419/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: **25 April 2021**
Expiration of Ethical Approval: **24 April 2022**

I wish to advise you that your application for recertification received on 15 April 2021 for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 08 June 2021.

Yours sincerely

……………………………………………………..
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

Appendix 4 Supplementary material to manuscript entitled "Affordable drug resistance genotyping of HIV-1 reverse transcriptase, protease and integrase genes, for resource limited settings"

SUPPLEMENTARY MATERIAL

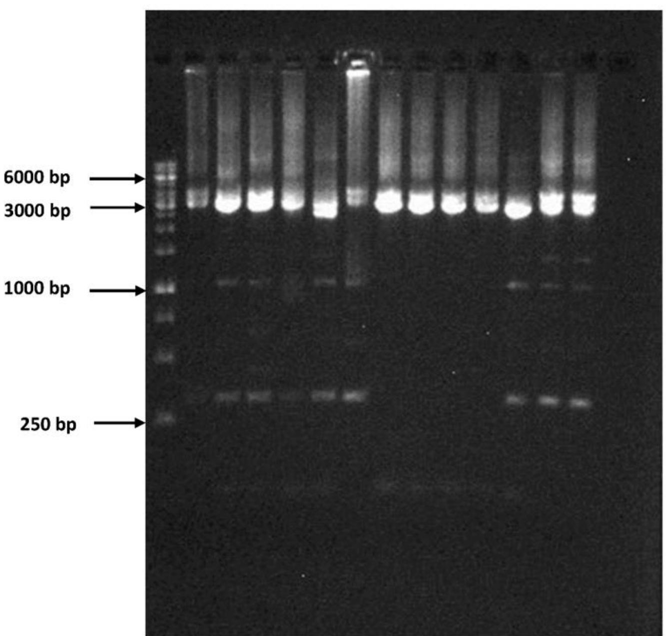

Figure S1 Gel image showing representation of amplicons after single-fragment PCR amplification

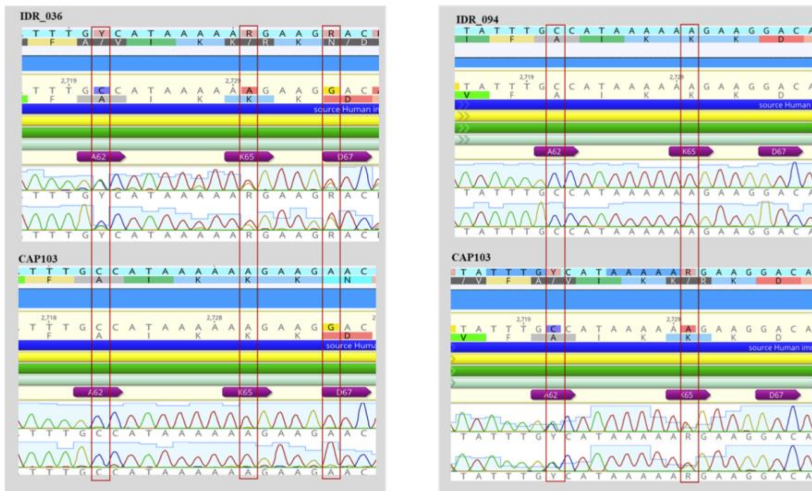

Figure S2 Comparison of discordant mutations between IDR and CAP103 sequences due to mixed bases

Note: In the first example (i.e. IDR036 on the left), the IDR sequence detected A62AV, K65KR and D67DN as mixtures that were not detected in CAP103 sequence. In the second example (i.e., IDR094 on the right), CAP103 sequence detected A62AV and K65KR mutations as mixtures that were not detected in the IDR sequence.

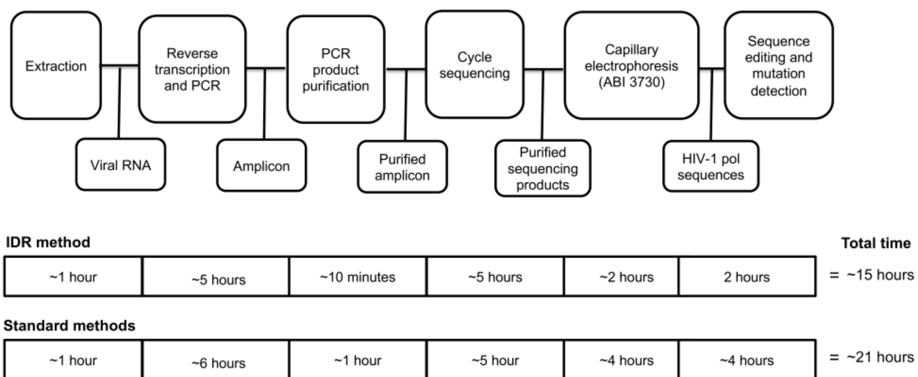

Figure S3 Comparison between IDR method and common in-house HIVDR genotyping workflows

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1_ RTC1F	Sample 2_ RTC1F	Sample 3_ RTC1F	Sample 4_ RTC1F								
B	Sample 1_ RTC2R	Sample 2_ RTC2R	Sample 3_ RTC2R	Sample 4_ RTC2R								
C	Sample 1_ RTC3F	Sample 2_ RTC3F	Sample 3_ RTC3F	Sample 4_ RTC3F								
D	Sample 1_ RTC4R	Sample 2_ RTC4R	Sample 3_ RTC4R	Sample 4_ RTC4R								
E	Sample 1_ KVL076	Sample 2_ KVL076	Sample 3_ KVL076	Sample 4_ KVL076								
F	Sample 1_ KVL082	Sample 2_ KVL082	Sample 3_ KVL082	Sample 4_ KVL082								
G	Sample 1_ KVL083	Sample 2_ KVL083	Sample 3_ KVL083	Sample 4_ KVL083								
H	Sample 1_ PAN2R	Sample 2_ PAN2R	Sample 3_ PAN2R	Sample 4_ PAN2R								

Figure S4 Sample and sequencing primer layout in 96-well reaction plate

Table S1 PCR primers used to amplify PR, RT and IN genes using a two-fragment approach

Primer (Direction)	Primer sequence	HXB2	Gene
MAW26 (Forward)	TCCCTCAGATCACTCTTTGGCAACGAC	2251 - 2277	PR and RT
RT21 (Reverse)	CTGTATTTCAGCTATCAAGTCCTTTGATGGG	3539 - 3509	PR and RT
Pro-1 (Forward)	TAGAGCCAACAGCCCCACCA	2147 - 2166	PR and RT
RT20 (Reverse)	CTGCCAATTCTAATTCTGCTTC	3462 - 3441	PR and RT
PANA3AF (Forward)	AGCATAGTAATATGGGGAAAGACTC	3684 - 3708	IN
5066R (Reverse)	ATCATCACCTGCCATCTGTTTTCCAT	5041 - 5066	IN

HXB2, nucleotide position of HIV-1 reference sequence; IN, integrase; PR, protease; RT, reverse transcriptase

Note: IN gene is amplified with only 2 primers using One-Step PCR

Table S2 Reverse transcription and first-round PCR conditions for amplifying PR and RT genes with two-fragment approach

First-round PCR Mastermix			
Reagent		**Volume per reaction (µl)**	**Concentration per reaction**
2X Reaction RT-PCR master mix		12.5	1X
Nuclease-free Water		2.25	-
MAW26 (5µM)		2.5	0.5µM
RT21 (5µM)		2.5	0.5µM
SSIV/ Platinum SuperFi DNA polymerase		0.25	0.02X
Total volume		**20**	-
Thermocycling conditions			
	Temperature (°C)	**Time**	**Cycle(s)**
cDNA synthesis	50	10 minutes	1
Pre-denaturation	98	2 minutes	1
Denaturation	98	10 seconds	
Annealing	56	20 seconds	40
Extension	72	2 minutes	
Final extension	72	10 minutes	1
Hold	4	∞	Hold

cDNA, complimentary DNA; RT-PCR, reverse transcription polymerase chain reaction; SSIV, SuperScript IV enzyme; µl, microliter; µM, micromolar; °C, Degrees Celsius

Table S3 Second-round PCR conditions for amplifying PR and RT genes with two-fragment approach

Second round PCR Mastermix			
Reagent		**Volume per reaction (µl)**	**Concentration per reaction**
Nuclease-free Water		18.4	-
10x PCR Buffer		2.5	1X
$MgCl_2$ (50mM)		1.0	2mM
dNTP (10mM)		0.5	0.2mM
Pro1 (5µM)		0.25	0.05µM
RT20 (5µM)		0.25	0.05µM
Platinum *Taq* DNA Polymerase		0.1	-
Total volume		**23**	-
Thermocycling Conditions			
	Temperature (°C)	**Time**	**Cycle(s)**
Pre-denaturation	94	2 minutes	1
Denaturation	95	10 seconds	
Annealing	56	20 seconds	40
Extension	72	2 minutes	
Final extension	72	10 minutes	1
Hold	4	∞	Hold

dNTP, deoxynucleoside triphosphate; $MgCl_2$, magnesium chloride; mM, millimolar; µl, microliter; µM, micromolar; °C, Degrees Celsius

Table S4 One-step RT-PCR conditions for amplifying the IN gene with two-fragment approach

One-step PCR Mastermix			
Reagent		**Volume per reaction (µl)**	**Concentration per reaction**
2X Reaction RT-PCR master mix		12.5	1X
Nuclease-free Water		1.5	-
PANA3AF (5µM)		2.5	0.5µM
5066R (5µM)		2.5	0.5µM
SSIV/ Platinum SuperFi DNA polymerase		1.0	0.08X
Total volume		**20**	-
Thermocycling Conditions			
	Temperature (°C)	**Time**	**Cycle(s)**
cDNA synthesis	50	10 minutes	1
Pre-denaturation	94	2 minutes	1
Denaturation	94	15 seconds	
Annealing	56	30 seconds	35
Extension	68	2 minutes	
Final extension	68	2 minutes	
Hold	4	∞	Hold

cDNA, complimentary DNA; RT-PCR, reverse transcription polymerase chain reaction; SSIV, SuperScript IV enzyme; µl, microliter; µM, micromolar; °C, Degrees Celsius

Table S5 Summary of all 96 samples processed and sequence outcome

IDR ID	Sex	Age	Log_{10} viral load copies/mL	Comments
IDR_001	Male	35	4,02	GSQ
IDR_002	Male	41	4,86	GSQ
IDR_003	Female	23	4,66	GSQ
IDR_004	Male	18	4,03	GSQ
IDR_005	Male	46	5,54	GSQ
IDR_006	Female	43	3,60	GSQ
IDR_008	Male	43	5,55	GSQ
IDR_013	Male	25	4,48	GSQ
IDR_014	Female	25	6,51	NAO
IDR_015	Male	47	3,88	GSQ
IDR_016	Female	39	3,31	GSQ
IDR_017	Male	20	4,45	GSQ
IDR_018	Female	36	4,40	GSQ
IDR_019	Female	30	3,81	GSQ
IDR_020	Female	30	4,17	GSQ
IDR_021	Female	30	4,54	GSQ
IDR_024	Male	53	4,20	GSQ
IDR_025	Female	33	3,16	NAO
IDR_026	Male	49	4,89	GSQ
IDR_027	Female	27	5,17	GSQ
IDR_028	Female	29	3,70	NAO
IDR_029	Male	40	3,17	NAO
IDR_030	Female	26	4,48	GSQ
IDR_031	Male	39	6,35	GSQ
IDR_033	Male	22	3,11	GSQ
IDR_034	Male	46	4,81	GSQ
IDR_036	Female	27	3,55	GSQ
IDR_038	Female	47	4,07	GSQ
IDR_039	Female	31	5,69	GSQ
IDR_040	Female	40	4,17	GSQ
IDR_041	Male	48	5,10	GSQ
IDR_043	Male	49	3,13	GSQ
IDR_045	Male	35	4,70	GSQ
IDR_046	Male	46	3,84	GSQ
IDR_048	Male	27	5,26	GSQ
IDR_049	Female	41	3,16	GSQ
IDR_050	Male	34	4,06	GSQ
IDR_051	Female	41	5,00	GSQ
IDR_055	Female	40	3,78	GSQ
IDR_056	Male	39	5,21	NAO
IDR_057	Male	67	5,86	GSQ
IDR_058	Female	46	3,41	GSQ
IDR_059	Female	21	5,36	GSQ
IDR_060	Male	51	5,59	GSQ
IDR_061	Male	37	4,37	GSQ
IDR_062	Male	33	3,91	GSQ
IDR_063	Male	30	3,45	GSQ
IDR_064	Male	36	4,80	GSQ
IDR_065	Female	35	4,46	GSQ
IDR_066	Female	47	5,09	GSQ

IDR_067	Male	46	4,07	GSQ
IDR_068	Female	41	4,82	GSQ
IDR_069	Male	43	4,74	GSQ
IDR_070	Female	36	4,33	GSQ
IDR_071	Male	32	4,77	GSQ
IDR_072	Female	41	3,45	GSQ
IDR_073	Female	25	3,80	GSQ
IDR_074	Female	52	4,74	Sample mix-up
IDR_075	Male	36	4,58	GSQ
IDR_076	Female	45	5,34	Sample mix-up
IDR_077	Male	50	3,05	NAO
IDR_078	Male	42	4,27	GSQ
IDR_079	Female	48	4,01	GSQ
IDR_080	Male	42	4,34	GSQ
IDR_081	Male	53	3,28	GSQ
IDR_082	Male	55	6,25	GSQ
IDR_083	Female	36	4,41	GSQ
IDR_084	Male	37	3,74	GSQ
IDR_085	Female	49	5,17	PSQ
IDR_086	Female	30	4,57	GSQ
IDR_087	Male	58	5,57	GSQ
IDR_088	Female	39	4,53	GSQ
IDR_089	Male	32	3,98	PSQ
IDR_090	Male	53	4,40	GSQ
IDR_091	Female	44	3,02	GSQ
IDR_092	Female	37	3,24	GSQ
IDR_093	Male	46	4,75	GSQ
IDR_094	Male	30	4,17	GSQ
IDR_095	Male	44	4,64	GSQ
IDR_096	Female	32	3,69	GSQ
IDR_097	Female	41	3,07	PSQ
IDR_098	Male	41	5,50	GSQ
IDR_099	Male	46	3,31	GSQ
IDR_100	Female	49	4,20	PSQ
IDR_101	Female	25	3,89	NAO
IDR_102	Female	23	5,43	GSQ
IDR_103	Female	32	3,20	NAO
IDR_104	Female	52	4,16	NAO
IDR_105	Male	20	5,27	PSQ
IDR_107	Male	29	6,22	GSQ
IDR_109	Male	35	4,01	PSQ
IDR_110	Female	38	5,53	GSQ
IDR_112	Female	37	3,91	GSQ
IDR_113	Male	44	6,62	GSQ
IDR_114	Female	47	3,45	GSQ
IDR_115	Female	30	6,28	PSQ

GSQ, good sequence quality; NAO, no amplification obtained; PSQ, poor sequence quality

Table S6 Details of discordant mutations resulting in discordant phenotypic predictions between IDR and CAP103 sequence pairs

		Protease		Reverse transcriptase		Discordant phenotypic predictions		Mutation score		Predicted regimen of choice	
IDR ID	**Line regimen**	**CAP103**	**IDR**	**CAP103**	**IDR**	**CAP103**	**IDR**	**CAP103**	**IDR**	**CAP103**	**IDR**
017	TDF+XTC+EFV	None	None	K65R,L74LI, M184V	K65R,**K70KT**, L74LI,M184V	ABC: H	ABC: H	105	120	AZT+3TC+DTG	AZT+3TC+DTG
						AZT: S	AZT: S	-25	-25		
						FTC: H	FTC: H	90	100		
						3TC: H	3TC: H	90	100		
						TDF: **I**	TDF: **H**	55	80		
020	AZT+XTC+LPVr	M46I,L76V	M46I,**I47IV**, L76V	M184V	M184V	ATVr: **PL**	ATVr: **L**	10	20	TDF+3TC+DTG	TDF+3TC+DTG
						DRVr: **L**	DRVr: **I**	20	30		
				K103N,Y188L	K103N,Y188L	LPVr: **I**	LPVr: **H**	50	65		
021	TDF+XTC+EFV	None	None	**K70KEQ**, M184V	M184V	ABC: **I**	ABC: **L**	30	15	TDF+3TC+DTG	TDF+3TC+DTG
						AZT: S	AZT: S	-20	-10		
						FTC: H	FTC: H	70	60		
				K103N,V106M E138A,V179L	K103N,V106M, E138A,V179L	3TC: H	3TC: H	70	60		
						TDF: **L**	TDF: **S**	15	-10		
036	TDF+XTC+EFV	None	None	**M41L**,D67N,**K70R**, M184V,K219Q	**A62V**,**K65R**, D67DN,M184V, K219KQ	ABC: H	ABC: H	65	75	TDF+3TC+DTG	AZT+3TC+DTG
						AZT: **H**	AZT: **S**	70	5		
						FTC: H	FTC: H	70	95		
						3TC: H	3TC: H	70	95		
				V106M,V179D	V106M,V179D, **F227FL**	TDF: **L**	TDF: **H**	20	65		
						DOR: **I**	DOR: **H**	50	100		
						EFV: H	EFV: H	90	105		
						ETR: PL	ETR: PL	10	10		
						NVP: H	NVP: H	90	120		
						RPV: L	RPV: L	25	25		
043	TDF+XTC+DTG +LPVr+ETR	M46I	M46I	**M41ML**,L210W, T215F,K219R	M41L,L210W, T215F,K219R	ATVr: PL	ATVr: PL	10	10	TDF+3TC+DRV +DTG	TDF+3TC+DRV+ DTG
						DRVr: **S**	DRVr: **S**	5	0		
		L10LF	None			LPVr: **L**	LPVr: **PL**	15	10		
				K103N,Y181C, P225H	K103N,Y181C, P225H						

045	AZT+XTC+LPVr	None	None	K65R,V75M,	K65R,V75M,	DRVr: **I**	DRVr: **H**	30	60	TDF+3TC+DTG	TDF+3TC+DTG
				M184V	M184V	EFV: H	EFV: H	120	180		
						ETR: **PL**	ETR: **I**	10	40		
				K103N,G190S	**L100LI**,K103N,	NVP: H	NVP: H	120	180		
					G190S	RPV: L	RPV: H	15	75		
048	TDF+XTC+EFV	None	None	K103N,G190GA	K103KN,	DOR: **S**	DOR: **I**	0	50	TDF+3TC+DTG	TDF+3TC+DTG
					V106VM,G190A	EFV: H	EFV: H	105	165		
						ETR: PL	ETR: PL	10	10		
				None	None	NVP: H	NVP: H	120	180		
						RPV: L	RPV: L	15	15		
062	TDF+XTC+EFV	None	**M46L**	K65R,M184V,	K65R,M184V,	ATVr: **S**	ATVr: **PL**	0	10	AZT+3TC+DTG	AZT+3TC+DTG
				K219E	K219E	DRVr: S	DRVr: S	0	0		
						LPVr: **S**	LPVr: **PL**	0	10		
				L100I,K103N,	L100I,K103N,						
				M230L	M230L						
067	TDF+XTC+EFV	None	None	**D67DN**,**L74LI**,	**D67G**,**K70E,**	ABC: **H**	ABC: **I**	65	35	TDF+3TC+DTG	TDF+3TC+DTG
				M184V	M184V	AZT: S	AZT: S	5	-10		
						FTC: H	FTC: H	60	70		
				K103N,V106M	K103N,V106M	3TC: H	3TC: H	60	70		
						TDF: **S**	TDF: **L**	0	20		
079	AZT+XTC+LPVr	None	None	M184V	**M41ML**,M184V	ABC: L	ABC: L	15	20	TDF+3TC+DTG	TDF+3TC+DTG
						AZT: S	AZT: S	-10	5		
						FTC: H	FTC: H	60	60		
				A98AG,K103N,	K103N,P225H	3TC: H	3TC: H	60	60		
				V108VI,P225H		TDF: S	TDF: S	-10	-5		
						DOR: **H**	DOR: **I**	60	30		
						EFV: H	EFV: H	130	105		
						ETR: **PL**	ETR: **S**	10	0		
						NVP: H	NVP: H	150	105		
						RPV: **L**	RPV:**S**	15	0		

080	AZT+XTC+LPVr	None	None	M184V	M184V	DOR: **L**	DOR: **S**	20	0	TDF+3TC+DTG	TDF+3TC+DTG
						EFV: H	EFV: H	120	90		
				K101KE,**K103KN**,	**K103S,**G190A	ETR: **I**	ETR: **PL**	30	10		
				G190A		NVP: H	NVP: H	150	120		
						RPV: **H**	RPV: **L**	60	15		
084	TDF+XTC+EFV	None	None	D67N,T69TADN,	D67N,K70R,	ABC: H	ABC: H	70	60	TDF+3TC+DTG	TDF+3TC+DTG
				K70KEGR,M184V,	M184V,K219Q	AZT: I	AZT: I	55	55		
				K219KQR		FTC: H	FTC: H	80	70		
						3TC: H	3TC: H	80	70		
						TDF: **I**	TDF: **L**	35	15		
				K103N,H221HY,	K103N,P225H,	DOR: I	DOR: I	45	30		
				P225HR,K238T	K238T	EFV: H	EFV: H	145	135		
						ETR: **PL**	ETR: **S**	10	0		
						NVP: H	NVP: H	150	135		
						RPV: **L**	RPV:**S**	15	0		
092	TDF+XTC+EFV	None	None	**D67DN**,M184V,	D67N,M184V,	ABC: L	ABC: L	25	25	TDF+3TC+DTG	TDF+3TC+DTG
				K219R	K219R	AZT: L	AZT: L	15	15		
						FTC: H	FTC: H	60	60		
				K103N,V108I,	K103N,V108I	3TC: H	3TC: H	60	60		
				F227FL		TDF: S	TDF: S	0	0		
						DOR: **H**	DOR: **L**	65	15		
						EFV: H	EFV: H	85	70		
						ETR: S	ETR: S	0	0		
						NVP: H	NVP: H	105	75		
						RPV:S	RPV:S	0	0		
093	AZT+XTC+LPVr	None	None	L74I,Y115F,	L74I,Y115F,	ABC: H	ABC: H	120	125	TDF+3TC+DTG	TDF+3TC+DTG
				M184V	M184V,**K219KE**	AZT: S	AZT: S	-10	0		
						FTC: H	FTC: H	60	60		
				V106M,H221Y,	V106M,H221HY,	3TC: H	3TC: H	60	60		
				F227C	F227C	TDF: **PL**	TDF: **L**	10	15		

094	TDF+XTC+EFV	None	None	**A62AV,K65KR,** M184V,**K219KN** K103N,V106M	M184V K103N,V106M	ABC: **H** AZT: S FTC: H 3TC: H TDF: **H**	ABC: **L** AZT: S FTC: H 3TC: H TDF: **S**	70 -10 95 95 60	15 -10 60 60 -10	AZT+3TC+DTG	TDF+3TC+DTG
095	AZT+XTC+LPVr	None	None	M184V K103N,H221Y, M230L	**K70KE,**M184V K103N,H221Y, M230L	ABC: **L** AZT: S FTC: H 3TC: H TDF: **S**	ABC: **I** AZT: S FTC: H 3TC: H TDF: **L**	15 -10 60 60 -10	30 -20 70 70 15	TDF+3TC+DTG	TDF+3TC+DTG
096	TDF+XTC+EFV	None	None	D67N,K70R, **L74I,**M184V K103N,V106M	D67N,K70R, M184V,**K219Q** K103N,V106M	ABC: H AZT: I FTC: H 3TC: H TDF: **S**	ABC: H AZT: I FTC: H 3TC: H TDF: **L**	70 35 60 60 5	60 55 70 70 15	TDF+3TC+DTG	TDF+3TC+DTG
099	AZT+XTC+LPVr	None	None	**A98AG** M184V	None M184V	DOR: **L** EFV: **L** ETR: **PL** NVP: **I** RPV: **L**	DOR: **S** EFV: **S** ETR: **S** NVP: **S** RPV: **S**	15 15 10 30 15	0 0 0 0 0	TDF+3TC+DTG	TDF+3TC+DTG

3TC, lamivudine; ABC, abacavir; ATV, atazanavir; AZT, zidovudine; DOR, doravirine; DRVr, ritonavir-boosted darunavir; DTG, dolutegravir; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine; H, high-level resistance; I, intermediate resistance; L, low-level resistance; LPVr, ritonavir-boosted lopinavir; NVP, nevirapine; PL, potential low-level resistance; RPV, rilpivirine; TDF, tenofovir; S, susceptible; XTC, lamivudine or emtricitabine

Table S7 Approximate cost for genotypic testing using IDR single-fragment approach

	Price ($US)	Number of samples	Price per sample ($US)	Total used	Total cost ($US)
RNA Extraction					
NucliSENS EasyMAG Kit	2598,70	1000	2,60	1	2,60
PCR					
SuperScript IV One-Step RT-PCR System (50µl)	824,27	200	4,12	1	4,12
Platinum Taq DNA Polymerase (120 reactions)	105,83	240	0,44	1	0,44
10mM dNTP Mix (0.2mL)	19,01	400	0,05	1	0,05
PCR Primers	13,33	700	0,02	2	0,04
Nuclease-Free Water (500mL)	46,82	3400	0,01	1	0,01
Gel electrophoresis					
TopVision Agarose Tablets (500mg x 200)	84,71	1000	0,08	1	0,08
UltraPure TBE Buffer, 10X (1L)	17,66	1000	0,02	1	0,02
SYBR Safe DNA Gel Stain (400µl)	70,27	400	0,18	1	0,18
GeneRuler 1kb DNA Ladder (2mL)	42,01	3300	0,01	1	0,01
6X Orange DNA Loading Dye (5mL)	28,51	330	0,09	1	0,09
PCR product purification					
ExoSAP-IT Express PCR Product Cleanup Reagent	237,90	480	0,50	1	0,50
Sequencing and analysis					
BigDye Terminator v3.1 Cycle Sequencing Kit (100 reactions)	1168,20	250	0,58	1	4,67
BigDye XTerminator Purification Kit (1000 reactions)	1346,40	250	0,67	1	5,39
POP-7 Polymer (25mL)	1578,60	8000	0,20	8	1,58
3730 Running Buffer, 10X (500mL)	232,50	200	1,16	1	1,16
Sequencing Primers	13,33	11250	0,00	8	0,01
Consumables					
1000µl tips (960)	132,09	960	0,14	6	0,83
200µl tips (960)	168,21	960	0,18	31	5,43
20µl tips (960)	112,14	960	0,12	5	0,58
10µl tips (960)	168,28	960	0,18	46	8,06

MicroAmp 8-Tube Strip with Caps, 0.2 mL	101,96	1000	0,10	3	0,31
1.5mL Nuclease-Free Eppendorf Tubes	63,13	500	0,13	5	0,63
50mL Falcon Tubes	132,47	25	5,30	1	5,30
MicroAmp Optical 96-Well Reaction Plates (10)	56,13	960	0,06	8	0,47
Adhesive PCR Plate Foils (100)	69,59	1000	0,07	1	0,07
Total					**$US42,62**

dNTP, deoxynucleoside triphosphate; g, gram; kb, kilobase; mL, millilitre; mM, millimolar; PCR, polymerase chain reaction; µl, microliter; µM, micromolar; $US, United States Dollar

Note: The exchange rate used was ~$1 US Dollar equivalent to ZAR15 South African Rands

Table S8 Approximate cost for genotypic testing using IDR two-fragment approach

	Price ($US)	Number of samples	Price per sample ($US)	Total used	Total cost ($US)
RNA Extraction					
NucliSENS EasyMAG Kit	2598,70	1000	2,60	1	2,60
PCR					
SuperScript IV One-Step RT-PCR System (50µl)	824,27	150	5,50	1	5,50
Platinum Taq DNA Polymerase (120 reactions)	105,83	240	0,44	1	0,44
10mM dNTP Mix (0.2 mL)	19,01	400	0,05	1	0,05
PCR Primers	13,33	700	0,02	2	0,04
Nuclease-Free Water (500mL)	46,82	3300	0,01	1	0,01
Gel electrophoresis					
TopVision Agarose Tablets (500mg x 200)	84,71	1000	0,08	1	0,08
UltraPure TBE Buffer, 10X (1L)	17,66	1000	0,02	1	0,02
SYBR Safe DNA Gel Stain (400µl)	70,27	400	0,18	1	0,18
GeneRuler 1kb DNA Ladder (2mL)	42,01	3300	0,01	1	0,01
6X Orange DNA Loading Dye (5mL)	28,51	330	0,09	1	0,09
PCR product purification					
ExoSAP-IT Express PCR Product Cleanup Reagent	237,90	480	0,50	1	0,50
Sequencing and analysis					
BigDye Terminator v3.1 Cycle Sequencing Kit (100 reactions)	1168,20	250	0,58	1	4,67
BigDye XTerminator Purification Kit (1000 reactions)	1346,40	250	0,67	1	5,39
POP-7 Polymer (25mL)	1578,60	8000	0,20	8	1,58
3730 Running Buffer, 10X (500mL)	232,50	200	1,16	1	1,16
Sequencing Primers	13,33	11250	0,00	8	0,01
Consumables					
1000µl tips (960)	132,09	960	0,14	6	0,83
200µl tips (960)	168,21	960	0,18	34	5,96

20µl tips (960)	112,14	960	0,12	6	0,70
10µl tips (960)	168,28	960	0,18	64	11,22
MicroAmp 8-Tube Strip with Caps, 0.2mL	101,96	1000	0,10	4	0,41
1.5mL Nuclease-Free Eppendorf Tubes	63,13	500	0,13	6	0,76
50mL Falcon Tubes	132,47	25	5,30	1	5,30
MicroAmp Optical 96-Well Reaction Plates (10)	56,13	960	0,06	8	0,47
Adhesive PCR Plate Foils (100)	69,59	1000	0,07	1	0,07
Total					**$US48,52**

dNTP, deoxynucleoside triphosphate; g, gram; kb, kilobase; mL, millilitre; mM, millimolar; PCR, polymerase chain reaction; µl, microliter; µM, micromolar

Note: The exchange rate used was ~$1 US Dollar equivalent to ZAR15 South African Rands