Published in final edited form as:

J Virol Methods. 2014 December 15; 210: 36–39. doi:10.1016/j.jviromet.2014.09.005.

Optimization of the Oligonucleotide Ligation Assay for the detection of nevirapine resistance mutations in Zimbabwean Human Immunodeficiency Virus type-1 subtype C

J. Mutsvangwa^{1,2}, I. A. Beck³, L. Gwanzura^{1,2}, M.T. Manhanzva², L. Stranix-Chibanda², T. Chipato², and L.M. Frenkel^{3,4}

I. A. Beck: ingrid.beck@seattlechildrens.org; L. Gwanzura: gwanzura@mweb.co.zw; M.T. Manhanzva: mnlsnmhznv2@gmail.com; L. Stranix-Chibanda: lynda@uz-ucsf.co.zw; T. Chipato: tchipato@zol.co.zw; L.M. Frenkel: lfrenkel@u.washington.edu

¹Biomedical Research & Training Institute (BRTI), Zimbabwe

²University of Zimbabwe, College of Health Sciences Zimbabwe

³Seattle Children's Research Institute, Seattle, WA, USA

⁴University of Washington Seattle, WA, USA

Abstract

An Oligonucleotide Ligation Assay (OLA) designed to detect Human Immunodeficiency Virus type-1 (HIV)-drug-resistance to the nevirapine (NVP) selected mutations K103N, Y181C, V106M and G190A was used to evaluate 200 archived dried blood spots (DBS) from infected infants participating in the Zimbabwean Early Infant Diagnosis (EID) Program. Consensus sequencing of specimens with indeterminate OLA results was performed to identify genetic sequence polymorphisms that appeared to compromise performance of the OLA. When consistent patterns of polymorphisms were observed the probes were redesigned, and DBS specimens with indeterminate OLA results were retested with the new Zimbabwe-specific (ZW) probes. OLA results obtained in Zimbabwe were compared to repeat testing in a US reference laboratory. 188/200 (94%) DBS yielded PCR amplification of HIV *pol*. ZW probes reduced indeterminate

Corresponding author: Junior Mutsvangwa, Biomedical Research and Training Institute, 10 Seagrave, Avondale, P O Box CY, 1753 Causeway, Harare, Zimbabwe, Phone: +263 4 703522, Mobile: +263 77 2 128 049, Fax : +263 4 333 464, jmutsvangwa@brti.co.zw and jnr.mutsvangwa@gmail.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The study was done as part fulfillment of Doctor of Philosophy degree for Junior Mutsvangwa, University of Zimbabwe, College of Health Sciences and it has previously been presented in part at the (1) National HIV and Conference: theme-Eliminating New HIV infections in Children: 5th September 2011 (2) Zimbabwe Annual Scientific Conference 11th July 2012: session 6 (3) BIT's 4th World DNA and Genome Day - Nanjing China 25th April 2013: Session WDD10,

Conflict of Interest Statement

The authors declare no conflicts of interest associated with this work.

Ethical Approvals

Consent to access the DBSs was given by the National Director of Laboratory Services, Ministry Of Health and Child Care (MoHCC) Zimbabwe because the samples were part of a routine MoHCC, the EID. The study was approved by the Biomedical Research and Training Institute (BRTI) Institutional Review Board (IRB): Ref AP88/10 and the Medical Research Council of Zimbabwe (MRCZ): Ref. MRCZ/B/127 and Seattle Children's Institutional Review Board, IRB #13781.

OLA results from 5.2% to 2.8% of codons evaluated ($p=0.02$), with 98.2% concordance between results obtained in the Zimbabwean and US laboratories. Optimization of OLA probes to accommodate polymorphisms in regional HIV variants improved OLA performance, and comparison to the USA results showed successful implementation of the OLA in Zimbabwe for detection of NVP resistance mutations in DBS specimens.

Keywords

HIV; HIV drug-resistance; oligonucleotide ligation assay; point mutation; genotyping; nevirapine

In Zimbabwe, NVP has been utilized widely as prophylaxis for prevention of mother-to-child-transmission of HIV (**PMTCT**) and/or as part of first-line antiretroviral regimens (**ART**) (Kuonza et al., 2010); (Zimbabwe, United Nations 2008 to 2009; Zimbabwe, PMTCT Report, 2010). Despite numerous scientific reports of selection of nevirapine (**NVP**)-resistance mutations, (Eshleman et al., 2005a; Eshleman et al., 2005b; Halvas et al., 2010; Johnson et al., 2005; Martinson et al., 2007; Micek et al., 2010) data from Zimbabwean infants are sparse. These data are unavailable due primarily to the unaffordable cost of consensus sequencing, the sequencing method used most commonly to assess HIV drug resistance (HIV-DR) (Beck et al., 2002; Bennett et al., 2008).

An economical oligonucleotide ligation assay (**OLA**) was developed to detect mutations generally associated with HIV-DR to NVP (K103N, Y181C, V106M and G190A) and other antiretrovirals (**ARVs**) (Ellis et al., 2004; Wallis et al., 2005; Micek et al., 2010). Ligation confers 100% specificity to the assay, but inter- and intra-subtype genetic polymorphisms with mismatches within two bases of the ligation site of the oligonucleotide probes result in indeterminate reactions (Beck et al., 2008; Wallis et al., 2005). Therefore, to detect regional HIV polymorphisms within new geographical locations, the oligonucleotide probes should be redesigned to accommodate HIV polymorphisms common in the target population (Edelstein et al., 1998; Beck et al., 2002; Beck et al., 2008; Jallow et al., 2007).

The objective of this study was to transfer the OLA to a Zimbabwean laboratory, optimize OLA reagents to detect the four NVP-resistance mutations (K103N, Y181C, V106M and G190A) in Zimbabwean infants, and validate the performance of the Zimbabwe-specific OLA reagents in the Zimbabwean laboratory for potential use in the detection of NVP resistant mutations in infants infected through MTCT.

The OLA for detection of NVP-associated HIV mutations was set-up in the Zimbabwean laboratory by its developers using supplies validated in Seattle, and Zimbabwean personnel were provided hands-on instructions. Following approval of the Zimbabwean Ethics Panel and Seattle Institutional Review Board, infant dried blood spots (DBS) were evaluated. All 200 DBS were from infants with documented perinatal treatment of the mother/infant pair with single-dose-NVP prophylaxis. The median interval between birth and DBS collection was 10 weeks (range 1–71 weeks, IQR: 6 and 16.5 weeks respectively).

DNA was extracted from the DBS using Chelex-100 (Beck et al., 2008) and HIV *pol* was amplified by nested PCR and tested with OLA probes for NVP-associated mutations

K103N, Y181C, V106M and G190A (Micek et al., 2010) designed using HIV subtype C sequences published in the Los Alamos National Laboratory (LANL) HIV database (<http://www.hiv.lanl.gov/content/index>) and are referred to in this paper as “generic probes” (Micek et al., 2010). The OLA conditions are described in the OLA Manual version 1.4, July 2007 (Beck et al., 2008). Mixtures of plasmids with mutant or wild-type sequence at the position of interest, combined to mutant concentrations of 0, 2, 5 or 100%, were assayed in each OLA plate. OLA reactions with optical densities (OD) 2% mutant control were considered mutant positive (Beck et al., 2002).

In total, 200 DBSs were processed for nested PCR. Amplicon was visualized in an agarose gel from 188 (94%) and 12 (6%) failed amplification. OLA using the generic subtype-C probes yielded valid results for 713 codons (94.8%) and 39 (5.2%) codons had indeterminate results, i.e., negative for both the mutant and wild type OLA reactions. Codon 181 had the highest proportion of indeterminate results (22/188; 11.7%), followed by codon 190 (3.7%), 103 (3.2%) and 106 (2.1%) (Table 1).

Thirty-eight HIV amplicons were sequenced (GenBank accession number KJ158204-KJ158241) in Seattle to identify genetic polymorphisms that were likely to interfere with the ligation of the oligonucleotide probes. When multiple Zimbabwean infants' sequences showed patterns of mismatches to the generic probes, new probes were synthesized to improve annealing to Zimbabwean HIV amplicons with polymorphisms adjacent to or within codons 103 and 181 (Table 2). Specimens evaluated with the new reagents included the 28 that tested indeterminate at codons 103 and 181 plus 26 that had yielded valid OLA results using the generic probes. The Zimbabwe-specific probes reduced the overall indeterminate rate from 5.2% to 2.8% ($p=0.02$), and detected additional mutants at codons 103 ($n=6$) and 181 ($n=4$) (Table 1).

Using the valid results from the generic probes plus the results obtained using the Zimbabwe-specific OLA probes, the number of infants with NVP resistance mutations detected increased from 41/188 (21.8%) to 48/188 (25.5%), with a single mutation in 39/188 (20.7%) and 2 mutations in 9/188 (4.8%). Resistance mutations were detected at similar rates from 0 through 24 weeks of age; but too few infants were tested at older ages to assess whether mutants decayed (Figure 1).

An inter-laboratory comparison of OLA results between Zimbabwe and Seattle laboratories was performed using the first 40 DBS evaluated in Zimbabwe. Concordant OLA results between the two laboratories were as follow: K103N (100%), V106M (39/40; 97.5%); Y181C, (38/40; 95%), and G190A (100%). The discrepant result at codon 106 was indeterminate in Zimbabwe and a mixture of mutant and wild type in Seattle. The two specimens discrepant at codon 181 showed mixtures in Zimbabwe and indeterminate results in Seattle.

The OLA was effectively established, validated and optimized for application to clinical specimens in Zimbabwe. The high concordance of valid OLA results obtained in the Zimbabwe laboratory compared to the Seattle laboratory attests to the relative simplicity of the assay. The high proportion (188/200; 94%) of DBS yielding PCR amplicons and OLA

results demonstrates the feasibility of linking OLA to testing of DBS from Early Infant Diagnosis (EID) programs (Bennett et al., 2008; Bertagnolio et al., 2010). The observation that a subset of infants between 6 weeks and 6 months of age had viral populations with 100% of the HIV variants encoding NVP-resistance, suggests reservoirs of NVP-resistance may persist, and could diminish the efficacy of future NNRTI-based ART regimens.

This study has several limitations. The factors contributing to non-amplification of HIV from 12/188 (6%) infants' DBS are unclear. The non-amplification could result from loss of sample integrity despite cold storage, low concentrations of HIV DNA or PCR primer-mismatches. In addition, only a limited proportion of infants' specimens, 54/188 (28.7%), were retested with the Zimbabwe-specific probes, and therefore the performance of these probes on the entire set of specimens is not known. Also, the probes for detection of V106M and G190A were not optimized for Zimbabwean HIV variants as indeterminate OLA results appeared to be caused by a variety of polymorphisms that could not be readily incorporated into new probes.

In summary, the OLA was implemented successfully in Zimbabwe. The optimization of the probes to local HIV polymorphisms showed a reduction in indeterminate reactions, confirming earlier annotations (Ellis et al., 2004; Wallis et al., 2005; Jallow et al., 2007; Beck et al., 2008; Micek et al., 2010), and increased the detection of drug resistant mutants at codons 103 and 181 by 25%. Use of this relatively low cost assay for detection of HIV drug-resistance in a low-resource environment could improve HIV care.

Acknowledgements

The authors would like to acknowledge the Ministry of Health and Child Care (MoHCC) Zimbabwe for approving use of DBS that remained following early HIV diagnosis by the EID Program, and for sharing of EID facilities. We also thank Professor David Katzenstein of Stanford University, USA, for his mentorship and Jennifer McKernan-Mullin of Seattle Children's Research Institute, Seattle, USA for submission of sequences to GenBank.

Funding

This work was supported by a Virology Developmental award to LMF from International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT) (1 U01 AI068632). The overall support for the IMPAACT was provided by the National Institute of Allergy and Infectious Diseases (U01 AI068632), the Eunice Kennedy Shriver National Institute of Child Health and Human Development, and the National Institute of Mental Health (AI068632). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

ARV	Antiretroviral
ART	Antiretroviral treatment
EID	Early Infant Diagnosis of HIV
HIV	Human Immunodeficiency Virus type-1
HIVDR	HIV Drug Resistance
NA	not applicable

NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitors
PCR	polymerase chain reaction
pMTCT	Prevention of Mother-to-Child Transmission
PI	Protease Inhibitors
ZW	Zimbabwe

References

- Beck IA, Crowell C, Kittoe R, Bredell H, Machaba M, Williamson C, Janssens W, Jallow S, van der Groen G, Shao Y, Jacob M, Samuel NM, de Rivera IL, Ngo-Giang-Huong N, Cassol S, Alemnji G, Frenkel LM. Optimization of the oligonucleotide ligation assay, a rapid and inexpensive test for detection of HIV-1 drug resistance mutations, for non-North American variants. *J. Acquir. Immune Defic. Syndr.* 2008; 48:418–427. PMC2583356. [PubMed: 18614915]
- Beck IA, Mahalanabis M, Pepper G, Wright A, Hamilton S, Langston E, Frenkel LM. Rapid and sensitive oligonucleotide ligation assay for detection of mutations in human immunodeficiency virus type 1 associated with high-level resistance to protease inhibitors. *J. Clin. Microbiol.* 2002; 40:1413–1419. PMID 11923366. PMC140364. [PubMed: 11923366]
- Bennett DE, Bertagnolio S, Sutherland D, Gilks CF. The World Health Organization's global strategy for prevention and assessment of HIV drug resistance. *Antivir Ther.* 2008; 13(Suppl 2):1–13. PMID 18578063. [PubMed: 18578063]
- Bertagnolio S, Parkin NT, Jordan M, Brooks J, Garcia-Lerma JG. Dried blood spots for HIV-1 drug resistance and viral load testing: A review of current knowledge and WHO efforts for global HIV drug resistance surveillance. *AIDS Rev.* 2010; 12:195–208. PMID 21179184. [PubMed: 21179184]
- Edelstein RE, Nickerson DA, Tobe VO, Manns-Arcuino LA, Frenkel LM. Oligonucleotide ligation assay for detecting mutations in the human immunodeficiency virus type 1 pol gene that are associated with resistance to zidovudine, didanosine, and lamivudine. *J. Clin. Microbiol.* 1998; 36:569–572. PMID 9466779. PMC104580. [PubMed: 9466779]
- Ellis GM, Mahalanabis M, Beck IA, Pepper G, Wright A, Hamilton S, Holte S, Naugler WE, Pawluk DM, Li CC, Frenkel LM. Comparison of oligonucleotide ligation assay and consensus sequencing for detection of drug-resistant mutants of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and plasma. *J. Clin. Microbiol.* 2004; 42:3670–3674. PMC497615. [PubMed: 15297515]
- Eshleman SH, Hoover DR, Chen S, Hudelson SE, Guay LA, Mwatha A, Fiscus SA, Mmiro F, Musoke P, Jackson JB, Kumwenda N, Taha T. Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after the administration of single-dose NVP. *J. Infect. Dis.* 2005a; 192:30–36. [PubMed: 15942891]
- Eshleman SH, Hoover DR, Chen S, Hudelson SE, Guay LA, Mwatha A, Fiscus SA, Mmiro F, Musoke P, Jackson JB, Kumwenda N, Taha T. Resistance after single-dose nevirapine prophylaxis emerges in a high proportion of Malawian newborns. *AIDS.* 2005b; 19:2167–2169. [PubMed: 16284468]
- Halvas EK, Wiegand A, Boltz VF, Kearney M, Nissley D, Wantman M, Hammer SM, Palmer S, Vaida F, Coffin JM, Mellors JW. Low frequency nonnucleoside reverse-transcriptase inhibitor-resistant variants contribute to failure of efavirenz- containing regimens in treatment- experienced patients. *J. Infect. Dis.* 2010; 201:672–680. PMC2835354. [PubMed: 20102272]
- Jallow S, Kaye S, Schutten M, Brandin E, Albert J, McConkey SJ, Corrah T, Whittle H, Vanham G, Rowland-Jones S, Janssens W. Development and evaluation of an oligonucleotide ligation assay for detection of drug resistance-associated mutations in the human immunodeficiency virus type 2 pol gene. *J. Clin. Microbiol.* 2007; 45:1565–1571. PMC1865891. [PubMed: 17329450]

- Johnson JA, Li JF, Morris L, Martinson N, Gray G, McIntyre J, Heneine W. Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. *J. Infect. Dis.* 2005; 192:16–23. [PubMed: 15942889]
- Kuonza LR, Tshuma CD, Shambira GN, Tshimanga M. Non-adherence to the single dose nevirapine regimen for the prevention of mother-to-child transmission of HIV in Bindura town, Zimbabwe: a cross-sectional analytic study. *BMC Public Health.* 2010; 10:218. PMC2873585. [PubMed: 20426830]
- Martinson NA, Morris L, Gray G, Moodley D, Pillay V, Cohen S, Dhlamini P, Puren A, Bhayroo S, Steyn J, McIntyre JA. Selection and persistence of viral resistance in HIV-infected children after exposure to single-dose nevirapine. *J. Acquir. Immune Defic. Syndr.* 2007; 44:148–153. [PubMed: 17117145]
- Micek MA, Blanco AJ, Beck IA, Dross S, Matunha L, Montoya P, Seidel K, Gantt S, Matediane E, Jamisse L, Gloyd S, Frenkel LM. Nevirapine resistance by timing of HIV type 1 infection in infants treated with single-dose nevirapine. *Clin. Infect. Dis.* 2010; 50:1405–1414. PMC3038662. [PubMed: 20384494]
- Wallis CL, Mahomed I, Morris L, Chidarikire T, Stevens G, Rekhviashvili N, Stevens W. Evaluation of an oligonucleotide ligation assay for detection of mutations in HIV-1 subtype C individuals who have high level resistance to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. *J. Virol. Methods.* 2005; 125:99–109. [PubMed: 15794978]
- Zimbabwe, United Nations General Assembly Special Session, Report on HIV and AIDS, January 2008 to December. 2009. http://data.unaids.org/pub/Report/2010/zimbabwe_2010_country_progress_report_en.pdf
- Zimbabwe, Prevention of Mother-to-Child Transmission (PMTCT) Report 2010. http://www.unicef.org/aids/files/Zimbabwe_PMTCTFactsheet_2010.pdf

Highlights

- The oligonucleotide ligation assay (OLA) can detect point mutations encoding HIV-drug-resistance.
- The ligation confers specificity, but depends on annealing of the probes to target sequences and complementary bases for two positions adjacent to each side of the ligation site.
- Genetic polymorphisms in target DNA can compromise ligation of probes, and significantly increase the proportion of indeterminate tests.
- Modifying probes to anneal to regional HIV genotypes can increase the proportion of valid assays.

Figure 1. Proportion of infants by age group with NVP-mutants detected by OLA using ZW-specific probes for K103N and Y181C together with generic probes for V106M and G190A
The proportion of 188 infants with HIV-drug resistance to nevirapine (NVP) or wild type virus is shown by the age when dried-blood-spot's were collected. All 188 infants were reported to have received a single dose of NVP within 48 hours of birth. Specimens were tested using an oligonucleotide ligation assay (OLA) for mutations conferring resistance to NVP at codons 103, 106, 181, and 190. The OLA was sensitive to 2% mutant variants in an infant's HIV viral population (i.e., quasispecies).

Annex 1   GenBank accession numbers for the nucleotide sequences

BankIt1692390 B40	KJ158204
BankIt1692390 E43	KJ158205
BankIt1692390 E49	KJ158206
BankIt1692390 F40	KJ158207
BankIt1692390 F39	KJ158208
BankIt1692390 B48	KJ158209
BankIt1692390 E39	KJ158210
BankIt1692390 F31	KJ158211
BankIt1692390 F37	KJ158212
BankIt1692390 B32	KJ158213
BankIt1692390 B36	KJ158214
BankIt1692390 B37	KJ158215
BankIt1692390 B42	KJ158216
BankIt1692390 B46	KJ158217
BankIt1692390 B47	KJ158218
BankIt1692390 B49	KJ158219
BankIt1692390 B51	KJ158220
BankIt1692390 B53	KJ158221
BankIt1692390 E31	KJ158222
BankIt1692390 E32	KJ158223
BankIt1692390 E33	KJ158224
BankIt1692390 E34	KJ158225
BankIt1692390 E35	KJ158226
BankIt1692390 E36	KJ158227
BankIt1692390 E37	KJ158228
BankIt1692390 E40	KJ158229
BankIt1692390 E41	KJ158230
BankIt1692390 E44	KJ158231
BankIt1692390 E45	KJ158232
BankIt1692390 E46	KJ158233
BankIt1692390 E47	KJ158234
BankIt1692390 E50	KJ158235
BankIt1692390 E51	KJ158236
BankIt1692390 F32	KJ158237
BankIt1692390 F33	KJ158238
BankIt1692390 F34	KJ158239
BankIt1692390 F35	KJ158240
BankIt1692390 F38	KJ158241

Figure 2.

OLA detection of NVP-resistant mutants in infants' DBS using Zimbabwe-specific probes (ZW) decreased indeterminate tests compared to "generic" HIV-1 subtype C probes

Table 1

Codon	Number (%) of specimens that OLA detected mutant, wild type, mixed or indeterminate genotype at each codon								Total number of codons tested
	100% Mutant		Mixture of mutant and wild-type		100% Wild Type		Indeterminate OLA		
	Generic ^a	ZW ^b	Generic	ZW	Generic	ZW	Generic	ZW	
	4 (2.1%)	7 (3.7%)	14 (7.5%)	17 (9.0%)	164 (87.2%)	162 (86%)	6 (3.2%)	2 (1.1%)	188
K103N	1 (0.5%)	1 (0.5%)	21 (11.1%)	25 (13.3%)	144 (76.6%)	154 (82%)	22 (11.7%)	8 (4.2%)	188
Y181C	0	NA ^c	8 (4.2%)	NA	176 (93.6%)	NA	4 (2.1%)	NA	188
V106M	0	NA	2 (1.1%)	NA	179 (95.2%)	NA	7 (3.7%)	NA	188
G190A	5 (0.7%)	8 (1.1%)	45 (6.0%)	52 (6.9%)	663 (88.2%)	671 (89.2%)	39 [*] (5.2%)	21 [*] (2.8%)	752
Totals, across all 4 codons									

^aGeneric OLA probes designed using HIV-1 subtype C sequences from Los Alamos database as of 2005

^bZW – Zimbabwe specific OLA probes modified from generic probes based on polymorphisms prevalent in Zimbabwean specimens initially testing indeterminate. Optimized probes were tested on a subset of specimens that included all specimens with indeterminate OLA results at codons 103 and 181.

^cNA- not applicable, as oligonucleotides probes for V106M and G190A were not redesigned; DBS specimens were only tested with generic OLA probes

^{*}Fisher's exact, two-tailed P value=0.02

Table 2

Modifications in generic HIV subtype C oligonucleotide ligation assay probes for Zimbabwean (*ZW*) sequences

<i>Generic 103 mut</i>	fluo-ACATCCCGCAGGGTTAAAAAAGAAC
<i>ZW specific 103 mut</i>	fluo-ACACCCAGCAGGGTTAAAAAAGAA <i>Y</i>
<i>Generic 103 wt</i>	Dig-ACATCCCGCAGGGTTAAAAAAGAAR
<i>ZW specific 103 wt</i>	Dig-ACACCCAGCAGGGTTAAAAAAGAAR
<i>Generic 103 common</i>	p-AAATCAGTRACAGTACTRGATGTGGG-bio
<i>ZW specific 103 com</i>	unmodified
<i>Generic 181 mut</i>	fluo-CAMAAAATCCAGAAATAGTCATCTG
<i>ZW specific 181 mut</i>	fluo-CACAAAAYCCAGAMATAGTCAT <i>Y</i> TG
<i>Generic 181 C wt</i>	dig-CACAAAATCCAGAAATAGTCATCTA
<i>ZW specific 181 wt</i>	dig-CACAAAAYCCAGAMATAGTCAT <i>Y</i> TA
<i>Generic 181 common</i>	p-TCAATACATGGATGATTTGTATGTA-bio
<i>ZW specific 181 com</i>	p-YCAATATATGGATGAC <i>T</i> TGTATGTA-bio

Bold italics denote modified bases to improve annealing and ligation to Zimbabwean HIV