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An Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings.

--Manuscript Draft--

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Abstract:	HIV-1 drug resistance has the potential to seriously compromise the effectiveness and impact of antiretroviral therapy (ART). As ART programmes in sub-Saharan Africa continue to expand, individuals on ART should be closely monitored for the emergence of drug resistance. Surveillance of transmitted drug resistance to track transmission of viral strains already resistant to ART is also critical. Unfortunately, drug resistance testing is still not readily accessible in resource limited settings, because genotyping is

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Question	**Response**

22 October 2013,

The Editor
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RE: Submission of our manuscript: **" An Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings "**

Dear Editor

We would like to thank you and the reviewers for the useful and positive comments. All of comments on the video and manuscript have been addressed and we trust that the paper and videos are ready for publication.

In certain instances (for example, question 1 and 2 of first reviewer), we followed the editorial suggestion to keep the paper succinct and did not edit the manuscript but provide a detailed answer to the reviewer.

We are pleased to resubmit the edited manuscript for your consideration.

We sincerely hope we have addressed all the comments to your satisfaction as well as the reviewers'.

Author contributions: Method development; JM, SD, PP, DK, TdO
Method optimization; JM, SD, SP, HM, CM, PP, TdO
Development of protocols; JM, SD, SP, HM, CM, PP, JV, RL, TdO
Manuscript preparation and Review; JM, SD, SP, PP, HM, CM, CS, RL, TFRdW, JV, DK, TdO
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Yours Sincerely

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An Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings

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Keywords: antiretroviral therapy; HIV-1; drug resistance; genotyping; affordable

Short Abstract:

Drug resistance testing for HIV-1 infected individuals failing antiretroviral therapy (ART) can guide future therapies and improve treatment outcomes. Optimising individual and population health outcomes in high HIV prevalence but resource-limited settings will ultimately require affordable and accessible drug resistance genotyping and interpretation methods.

Long Abstract:

HIV-1 drug resistance has the potential to seriously compromise the effectiveness and impact of antiretroviral therapy (ART). As ART programmes in sub-Saharan Africa continue to expand, individuals on ART should be closely monitored for the emergence of drug resistance. Surveillance of transmitted drug resistance to track transmission of viral strains already resistant to ART is also critical. Unfortunately, drug resistance testing is still not readily accessible in resource limited settings, because genotyping is expensive and requires sophisticated laboratory and data management infrastructure. An open access genotypic drug resistance monitoring method to manage individuals and assess transmitted drug resistance is described. The method uses free open source software for the interpretation of drug resistance patterns and the generation of individual patient reports. The genotyping protocol has an amplification rate of greater than 95% for plasma samples with a viral load >1,000 HIV-1 RNA copies/ml. The sensitivity decreases significantly for viral loads <1,000 HIV-1 RNA copies/ml. The method described here was validated against a method of HIV-1 drug resistance testing approved by the United States Food and Drug Administration (FDA), the Viroseq genotyping method. Limitations of the method described here include the fact that it is not automated and that it also failed to amplify the circulating recombinant form CRF02_AG from a validation panel of samples, although it amplified subtypes A and B from the same panel.

Introduction

The HIV epidemic in southern Africa has been evolving rapidly[1] with a concomitant exponential increase in individuals on antiretroviral therapy (ART), especially in South Africa[2,3]. As evidence on the epidemiologic impact of large scale treatment programmes in reducing incidence[4] and increasing life expectancy in resource-limited settings (RLS)[5] continues to accumulate, efforts to increase ART coverage will be intensified. The evolution of guidelines towards the use of treatment as a prevention tool[6,7] under the test and treat programmes means that the absolute number of individuals on treatment will further increase. Large numbers of individuals will be on ART for longer periods of time as the average life expectancy of individuals on ART nears that of the HIV uninfected population[8]. The development and transmission of HIV drug resistance has always been considered a

threat to the achievements of ART[9-12]. Thus, there is a need for more rigorous surveillance and monitoring of drug resistance as more individuals are initiated onto ART.

Genotypic drug resistance testing (GRT) has been used successfully in developed countries, both for surveillance as well as monitoring of HIV-1 drug resistance in individuals receiving ART. In these settings, GRT has been integrated into the continuum of care for HIV-1 infected individuals. Most international guidelines recommend GRT for adult or paediatric patients failing ART (first-line and second-line)[13-15], paediatric patients exposed to prevention of mother-to-child transmission (pMTCT) regimens but subsequently infected[16], and in settings with high-levels of transmitted drug resistance, among acutely infected individuals[13-15]. However, the cost, technology and infrastructure requirements have limited the implementation of similar approaches to drug resistance monitoring in RLS.

The South African HIV treatment and monitoring guidelines do not currently recommend the use of GRT in guiding choice of ART for individuals failing first-line regimens[17]. Individuals are switched based primarily on virological (HIV-1 RNA viral load) parameters. However in 2012, the Southern African HIV Clinicians Society published the first Southern African ARV drug resistance testing guidelines [18]. These guidelines recommend GRT testing for all adults failing first-line and second-line ART, and for infected infants and children exposed to pMTCT[18]. However, GRT is not recommended[18] for acutely infected individuals because there is no current evidence for high levels of transmitted drug resistance in southern Africa[19-29]. It is expected that some of these recommendations will be integrated over time into the national treatment and monitoring guidelines of the various countries in the region. Already, in the 2013 South African treatment guidelines there is now recommendation of GRT at time of second-line failure for adults and at time of first- or second-line PI-based regimen failure for children [30].

It has been shown that incorporating GRT into treatment guidelines in South Africa would be potentially cost-neutral. Considering the cost of the second line regimen drugs which are relatively more expensive that the first line drugs, using GRT to identify patients who truly need to be switched to second line therapy will not result any additional cost to the program. In addition, GRT can also identify other reasons for failure, conserve treatment options and generate information about emerging resistance patterns[31]. Therefore, it is necessary to reduce the cost of drug resistance monitoring methods even further in order to improve access, quality of care and outcomes.

Here, we present a GRT method designed to use generic (open source) primers for reverse transcription, polymerase chain reaction (PCR) and sequencing **(Table 2)**, as well as mostly open source software for drug resistance interpretation. For clinical management, the protocol is complimented by a comprehensive review and reporting method with specialist interpretation of the laboratory drug resistance report with close adherence to the national treatment guidelines. The protocol is divided into four different components; 1) HIV Ribonucleic Acid (RNA) Extraction, 2) Reverse Transcription & Polymerase Chain Reaction (PCR) amplification of viral targets, 3) Sequencing and 4) Bioinformatics methods for analysis of chromatograms, alignment, curation and interpretation of sequence data.

Protocol Text:

1 Ethylenediaminetetraacetic acid (EDTA) Whole Blood Processing Blood can be processed immediately after collection of can be stored at 4 °C for no more than 24 hours.

1.1 Working in a biosafety cabinet, allow the EDTA whole blood sample to reach room temperature.

1.2 For each sample, label enough cryovials with the sample identification (ID), storage material (plasma) and date.

1.3 Centrifuge the samples for 10 min at 1, 000 x g. Do not use brakes to stop centrifuge. This will yield three layers (from top to bottom): plasma, leucocytes (buffy coat) – a very thin layer - and erythrocytes, including platelets.

1.4 Carefully aspirate the supernatant (plasma) and aliquot 500 µl into each cryovial. Take care not to disrupt the cell layer (buffy coat) or transfer any cells.

1.5 Store at –80 °C until needed for RNA extraction or proceed to RNA exctraction immediately.

2 RNA Extraction

2.1 Prepare an extraction worksheet with the IDs of the samples to be extracted including positive and negative plasma controls.

2.2 For each sample to be extracted, label a 1.5 ml sterile microcentrifuge tube with the sample ID, extraction date and "RNA". Also label an assembled column and collection tube as well as a 2 ml microcentrifuge tube containing working lysis solution with corresponding numbers from the extraction worksheet.

2.3 Working in the Bio-Safety Cabinet, add 200 µl sample to the corresponding 2 ml microcentrifuge tube of working lysis solution.

2.4 Vortex well and incubate for 10 min at room temperature.

2.5 After 10 min, centrifuge the tube briefly.

2.6 Add 800 µl of absolute ethanol to each of the tubes.

2.7 Mix by pulse vortexing and briefly centrifuge.

2.8 Transfer 600 µl of this solution to the corresponding column/collection tube assembly. Centrifuge at 6, 000 x g for 1 min.

2.9 Transfer column to a new collection tube and discard the old collection tube containing the filtrate. Repeat the above step 2.8 (above) two more times.

2.10 Add 500 µl wash buffer AW1 to each column and centrifuge at 6, 000 x g for 1 min.

2.11 Discard the filtrate and collection tube and transfer the column to a new collection tube.

2.12 Add 500 µl was buffer AW2 and centrifuge at 20, 000 x g for 3 min. Repeat step 2.11.

2.13 Centrifuge in a new collection tube at 20, 000 x g for an additional 2 min.

2.14 Discard filtrate and place column in 1.5ml microcentrifuge tube.

2.15 Add 60 µl Buffer AVE (RNase free water) to the middle of the column ensuring that you do not dispense the liquid on the side of the column.

2.16 Incubate at room temperature for 1 min.

2.17 Centrifuge at 6, 000 x g for 2 min.

2.18 Discard the column and cap the 1.5 ml microcentrifuge tubes.

2.19 The samples are now ready for reverse transcription.

2.20 If testing is to be performed immediately, store at 4 °C for up to six hours. However, if testing is to be delayed then place at -80 °C immediately. NB: do not freeze/thaw the samples more than 3 times.

3 Reagent Preparation for Reverse Transcription

3.1 Before starting, calculate the volumes of each of the reagents required for the number of samples being processed including, the positive and negative plasma controls. Also add a reagent control.

3.2 Using the calculated volumes from 3.1 (above), prepare the deoxyribonucleotide triphosphate (dNTP)-primer mix in a clean, sterile 200 µl PCR tube followed by briefly pulse vortexing. Each sample should have 0.5 µl of the reverse primer RT21 and 0.5 µl of the dNTP, **see Table 3**.

3.3 Aliquot 1.0 µl of the dNTP-primer mix to 200 µl PCR tubes.

3.4 Prepare reverse transcriptase (RT) enzyme mix by adding 1 µl of the 10 X reverse transcription buffer, 1 µl of 0.1M DTT and 2 µl of 25mM $MgCl_2$ to a sterile tube followed by vortexing and briefly centrifuging, **see Table 4**.

3.5 Add 0.5 µl each of the enzymes RNAseOUT and Superscript III reverse transcriptase to the enzyme mix tube then tap the tube gently to mix.

3.6 Keep the tubes with the dNTP-primer mixes and enzyme mix on a cold block and move to the RNA station.

4 Reverse Transcription

4.1 Add 6 µl of the RNA sample to the dNTP-primer mix tube followed by briefly vortexing to mix.

4.2 After the addition of the RNA, move to the PCR room with both dNTP/primer/RNA mix and RT Enzyme mix tubes on a cold block or ice.

4.3 Briefly centrifuge the dNTP/primer/RNA mix tubes (from step 4.2) and place them into a thermocycler.

4.4 Heat at 65 °C for 5 min to denature the RNA.

4.5 Rapidly cool to 4 °C, hold for 2 min.

4.6 Pause the thermocycler while still at 4 °C; take out the tubes.

4.7 Quickly add 5 µl of the enzyme mix while keeping the tubes on a cold block.

4.8 Mix gently by tapping the tube then briefly centrifuge the tubes and return to the thermocycler.

4.9 Hold the tubes at 50 °C for 60 min to reverse transcribe the RNA followed by enzyme denaturation at 85 °C for 5 min to stop the reverse transcription.

4.10 Cool to 37 °C. As soon the temperature gets to 37 °C, pause and take the tube out of the thermocyler.

4.11 Quickly add 0.5 µl of RNAse H to the tubes and return to the thermocyler.

4.12 Hold at 37 °C for 20 min and then cool to 4 °C.

4.13 The complementary DNA (cDNA) can be used immediately or can be stored at -20 °C or colder until needed. However, the long term storage of cDNA should be at -80 °C.

5 Reagent Preparation for PCR

5.1 Before starting, calculate the volumes of each of the reagents required for the number of samples being processed and the controls. In addition to the three controls (Positive, Negative, and Reagent), you can also add a PCR control (HIV DNA). The first and second round PCR mixes can be prepared simultaneously and the second master mix stored at -20 °C until needed. Mixes can be stored for approximately 8 hrs.

5.2 Add 18.4 µl water, 2.5 µl 10 X buffer, 1.0 µl $MgCl_2$, 0.5 µl dNTPs, and 0.25 µl of each of the primers as shown on **Table 5** and vortex.

5.3 Add 0.1 µl of Platinum Taq polymerase (5U/ µl) and gently mix the tube by tapping it.

5.4 Aliquot 23 µl of the master mix to 200 µl PCR tubes.

5.5 With the master mix tubes on a cold block or ice move to the PCR room.

6 Nested PCR

6.1 Add 2 µl of the cDNA to 23 µl of the 1st round PCR master mix.

6.2 Close the tubes, put the samples in the thermocycler and use the following PCR cycling conditions: 94 °C for 2 min, 30 cycles of 95 °C for 30 sec, 58 °C for 20 sec and 72 °C for 2 min, followed by a final extention at 72 °C for 10 min as shown on **Figure 1. (Place Figure 1 here).**

6.3 Continue to the 2nd round PCR stage or store the 1st round PCR products at –20 °C or colder until required at a later stage.

6.4 For 2nd round PCR, add 2 µl of the 1st round PCR product to 23 µl of the 2nd round PCR master mix and use the same PCR program on **Figure 1.**

7 Gel Electrophoresis

7.1 Gel preparation

7.1.1 Add a 0.5 g of agarose tablet to a 250 ml glass flask and add 50 ml of 1 X TBE buffer to the flask.

7.1.2 Heat in microwave to boiling; swirl frequently (approximately every 30 sec) until completely solubilized. Use a silicone grip or silicone oven glove to grasp the hot flask. The agarose solution can boil out of the flask very easily so closely monitor this process.

7.1.3 Cool at room temperature for approximately 10 min.

7.1.4 Pour agarose into a gel casting tray containing appropriate size comb; gel is ready to use in approximately 20-30 min.

7.1.5 Place gel in electrophoresis chamber and run as recommended by the manufacturer.

7.2 Gel electrophoresis and visualization.

7.2.1 Vortex Novel Juice for 10 sec prior to use.

7.2.2 Dilute 1 µl of Novel Juice with 5 µl of DNA sample and mix.

7.2.3 Dilute 3 µl of Novel Juice with 3 µl of molecular weight marker and mix.

7.2.4 Load the mixes from 7.2.2 and 7.2.3 (above) and run the gel at 100V and 400W for 40 min to evaluate the PCR amplification.

7.2.5 Positive amplification can be visualized under UV light as 1315 bp fragment, Figure 2. (Place Figure 2. here).

7.2.6 There should be no amplification in the Negative and reagent controls, thus indicating absence of contamination.

8 PCR Product clean up

8.1 In preparation for the sequencing reaction, the positive second round PCR products are cleaned up using the PureLink PCR purification kit.

8.2 Add 80 µl of working Binding buffer High-Cutoff (B3) to 20 µl of PCR product and pipette mix.

8.3 Add the sample mixed with the binding buffer to a spin column in a collection tube.

8.4 Centrifuge the column at 10, 000 x g for 1 min. Transfer the column into a new collection tube.

8.5 Wash the column with 650 µl of Wash Buffer with ethanol.

8.6 Centrifuge the column at 10, 000 x g for 1 min. Transfer the column into a new collection tube.

8.7 Centrifuge the column at maximum speed for 2-3 min to remove any residual wash buffer.

8.8 Place the spin column in a clean 1.7 ml elution tube supplied with the kit.

8.9 Add 40 µl of elution buffer to the center of the column and incubate the column at room temperature for 1 min.

8.10 Centrifuge the column at maximum speed for 2 min (>10, 000 x g).

8.11 The elution tube contains your purified PCR product ready for sequencing. Discard the column.

8.12 Determine the concentration and quality of the DNA using a Nanodrop.

8.13 If no in-house sequencing facilities are available, the purified PCR products can be sent to a commercial sequencing lab at this stage.

9 Sequencing Reactions

9.1 The PCR products are sequenced using the big dye terminator kit ver 3.1 and 4 primers for each sample (two forward and two reverse). The primer sequences are shown in **Table 2.** Therefore, after the sequencing run, each sample will have four sequences to be assembled into a contig.

9.2 Set up the sequencing reactions as indicated in **Table 6** for each of the four primers.

9.3 Mix the sequencing buffer and the primers by vortexing before use.

9.4 Mix the water, buffer and primer before the addition of the big dye sequencing. Mix by vortexing.

9.5 Gently mix the master mix after adding the big dye sequencing mix by inverting the tube or tapping it gently.

9.6 Aliquot 9 µl of the master mix into a 96 well optical plate.

9.7 In order to run 24 samples per plate, set up the plate as indicated below **Figure 3. (Place Figure 3. here).**

9.8 Add 1.0 µl of the DNA sample (~20-40 ng), cover the plate with an adhesive alluminium cover and then gently mix it.

9.9 Centrifuge at 3000 g for 1 min. Remove the aluminium cover and add a rubber sealing mat.

9.10 Place the plate on the thermocycler and run the following cycling programme shown in **Figure 4**. (Place Figure 4. here).

9.11 When the PCR finishes, clean up the sequencing product immediately.

10 Sequencing Clean up

10.1 For each sequencing reaction, mix 50 µl absolute ethanol and 5 µl 3M Sodium acetate.

10.2 Using a multi-channel pipette, add 55 µl of the Sodium acetate/EtOH solution to each well.

10.3 Seal wells with adhesive aluminium cover, ensuring that each well is sealed properly.

10.4 Centrifuge at 3000 x g for 20 min.

10.5 After 20 min, remove cover and invert the plate, in one smooth motion, onto a folded lab tissue (DO NOT bang to get rid of supernatant as this will dislodge the pellet!).

10.6 Centrifuge the inverted plate on the same tissue at 150 x g for 2 min.

10.7 Immediately add 150 µl COLD 70% EtOH. **DO NOT delay addition of ethanol at this step.**

10.8 Seal with the same adhesive aluminium cover and vortex.

10.9 Centrifuge at 3000 x g for 5 min.

10.10 Invert plate onto a new folded tissue and centrifuge inverted at 150 x g for 1 min.

10.11 After the centrifugation, place uncovered in thermocycler and dry it at 50 °C for 2 min.

10.12 Once the plates is dry, seal it with adhesive foil covers, wrap in foil and store at –20 ˚C until ready to proceed with the sequencing electrophoresis.

10.13 When ready to sequence, dissolve cleaned sequencing products in 10 µl Hi-Di formamide, denature and load for electrophoresis.

11 Bioinformatics

11.1 Sequence Assembly

11.1.1 Launch the program Geneious.

11.1.2 Create a working folder to store the sequences.

11.1.3 Import the ABI files generated by the sequencing machine to the working folder using the import tool. Geneious will allocate percentage quality score for each sequence imported.

11.1.4 Open sequences with quality scores >70% by double clicking on them.

11.1.5 Each file should open in a new window. The software will indicate the quality at each nucleotide position of the chromatogram of the sequence quality using light blue bars. The higher the bar, the better the quality of the base call.

11.1.6 Using the cursor, select the mid section of the sequence leaving out the ends, which are usually of poor quality.

11.1.7 Click on the extract button to extract the region with good quality sequence.

11.1.8 Select all four extracted sequences for each sample and assemble them against a reference sequence.

11.1.9 Inspect the assembled sequence to ensure that you are in the correct reading frame. If you are in the correct reading frame, the beginning of Protease should start with the following amino acids: PQILWT. The beginning of RT will start with PISPIE.

11.1.10 Extract the contig region covering the beginning of PR to the 300th RT codon. During this process, also check for insertions or deletions.

11.1.11 Go through the consensus sequence of the extracted contig, identifying any ambiguities and verify positions with mixed bases by inspecting quality (symmetry, height, background and shoulders of flanking regions) of the base calls.

11.1.12 Select the consensus sequence and click the extract button to create a separate file of the consensus sequence from the four primers and label it appropriately.

11.1.13 Export the sequence to a backup storage folder on the computer or a network folder.

11.2 Sequence Quality Assessment (HIVDB)

11.2.1 Analyze the sequence using the HIVDB program at http://hivdb.stanford.edu.

11.2.2 Check for deletions and insertions in the summary data and ensure that the sequence covers all the 99 protease (PR) codons and the 1st 300 RT codons.

11.2.3 Check for any highlighted quality assurance (QA) issues in both the PR and RT regions, such as stop codons, frame shifts, ambiguous positions and unusual residues.

11.3 Sequencing Quality Control

11.3.1 Blast the new sequence against a local sequence database from previous run.

11.3.2 If the new sequence is >97% similar to any sequence in the database, all the stages of the protocol should be reviewed, starting with the sequence analysis and going back to the RNA extraction to ensure that there were no mix ups (sample switching, mislabeling) or contamination.

11.3.3 If no problems are identified, repeat the analysis of both the old and new samples from the RNA extraction stage.

11.3.4 If the sequences are still >97% similar, review the patient history to assess for any epidemiologic linkage between the individuals.

11.4 Phylogenetic Analysis

11.4.1 Align all the sequences from the database using ClustalW programme in Geneious.

11.4.2 Manually check the alignment for misaligned sequences, deletions and insertions and edit accordingly.

11.4.3 Construct a phylogenetic tree using PHYML, Geneious tree builder or other tree builders in Geneious.

11.4.4 Examine the tree for samples with short branch lengths.

11.4.5 Review the samples with short branch lengths for possible contamination.

12 REGA DB Informatics

12.1 Sequence Upload

12.1.1 Log into the RegaDB using a unique username and password.

12.1.2 On the drop down menu, under Patient ID, select “Begins with”.

12.1.3 Add the patient ID and select the individual whose genotype is to be uploaded.

12.1.4 On the menu to the left, select “viral isolate”.

12.1.5 From the options under viral isolate select “add”.

12.1.6 Enter the Sample date, Sample I.D, Sequence I.D and Sequence date.

12.1.7 Select “choose file” and then navigate to the fasta file of the sequence to be uploaded.

12.1.8 After selecting the fasta file to be uploaded, click on upload.

12.1.9 Once the uploaded sequence appears in the nucleotide box under the sequence identifies and dates, click the ok button at the bottom right of the window.

12.1.10 Check for PR and RT protein alignment by clicking the button protein and selecting either PR or RT.

12.1.11 Check for the drug resistance mutation by clicking on the resistance button. This gives you the resistance profiles from three algorithms: ANRS, Stanford HIVDB and RegaDB.

12.2 **Report generation using REGA**

12.2.1 Log into the RegaDB using your unique username and password.

12.2.2 On the drop down menu, under Patient ID, select “Begins with”.

12.2.3 Add the patient ID and select the individual whose report is to be generated.

12.2.4 On the menu to the right, select viral isolate.

12.2.5 From the options under viral isolate click on “view”.

12.2.6 Double click on the viral isolate for which you want to create a report.

12.2.7 On the viral isolate window, click on the viral isolate report tab.

12.2.8 Select the algorithms for the interpretation of the genotype from the drop down menu and then select report template to use.

12.2.9 Once the algorithm and the template are selected, click on the button “generate”.

12.2.10 Download the rtf document generated.

12.2.11 Open the rtf document as a word document.

12.2.12 Resize the treatment history chart.

12.2.13 After the chart, add the section “Clinical chart and resistance interpretation”.

12.2.14 Using the data on the resistance table and the clinical chart, add a description of the patient's resistance profile starting with the patient's treatment history, and the drugs to which the viral isolate is resistant. Also add a description of the patient's viral load and CD4+ cell count profiles from the chart.

12.2.15 Send the report to the Infectious Diseases (ID) specialist for review and recommendations on future patient management. This process is also a very important quality assurance stage. Any errors in the genotype or inconsistences in the treatment history, virological and immunological profiles can be identified and reviewed before a final report is sent, with all the recommendations, to the clinician managing the patient.

Representative Results

The method validated was a modification of a previously reported method.[20] The Viroseq genotyping method, which has been approved by FDA, was used as the reference method in the validation. A panel of proficiency testing samples obtained from the French National Agencies for Research on AIDS and Viral Hepatitis (ANRS) was used in the primary comparison between the two methods. The two genotyping methods were 100% concordant in identifying all clinically important drug resistance-associated mutations as interpreted by the HIVDB programme for the samples that were successfully amplified by both methods. As shown in **Table 7**, the nucleotide sequences of the three pairs were ≥99.5% identical. The predicted amino acid sequences were 100% identical. One sample out of five could not be successfully amplified by Viroseq. In addition to the sample not amplified by Viroseq, the in-house method failed to amplify a second sample which was shown to be a circulating recombinant virus (CRF02_AG) by Viroseq. The three samples that amplified with both methodologies were subtype B (two samples) and subtype A (one sample). **(Place Table 7. here)** Figure 5. shows how the sequences cluster with each other and other reference sequences from the Los Alamos HIV sequence database in a Neighbour Joining phylogenetic tree.
(Place Figure 5. here)

A panel of five samples was used to assess the precision of the in-house method. Ten replicate genotypes were generated for each of the five samples. Using the 16 Capillary 3130xl genetic analyzer, 48 of the 50 genotypes were generated from 24 runs, prepared on the same day. For all five samples, the predicted amino acid sequences were 100% concordant amongst replicates. For the nucleic acid sequences, there was >99% paire-wise similarity.

During the first two years of the use of this method, sixty samples were repeated randomly from RNA extraction to sequencing. There were no statistically significant differences between the sequence quality score and the number of mixed bases between the replicates. Both the nucleotide and amino acid pairwise comparisons for the sixty pairs were greater than 99% identical. Thus the drug resistance mutations for all the pairs were 100% concordant.

Cost reduction

The reaction volumes for RT, PCR and sequencing were reduced by at least half, relative to the original method[20,32], without compromising on the quality of the sequences generated. This enabled a reduction in cost of 50% for the RT and PCR stages.

The new method was originally designed to work with six sequencing primer to sequence all 99 codons of the protease gene and the first 300 codons of the reverse transcriptase gene[20,32]. Similar methods also use six to eight primers[33,34]. Some recently published methods have used less than six primers, although sometimes sequencing the protease and RT genes seprately[35,36]. We sought to reduce the number of sequencing primers from six to four, (**Figure 6**)**.[Place Figure 6. here]** Sequences from a set of 17 samples generated from six primers were compared to sequences generated after exclusion of two primers

(MAW46 and RTY). The subtypes were 14 subtype C, two subtype B, and one subtype A. There were no significant differences in sequence quality scores. Again, the average pairwise identity between the 17 pairs of nucleic acid was 99% and 100% on the amino acid level. Thus, reducing the sequencing primers from six to four resulted in a reduction in the sequencing cost by almost a third.

The only proprietary software tool used in this protocol was Geneious for sequence assembly. The drug resistance interpretation tools, as well as the report generating tools are all free, open access tools. This reduces the cost further by eliminating the costs associated with the use of proprietary software. Further, collective negotiation allowed the reagents for this protocol to be packaged into a kit for easy access from Life Technologies and is available as the SATuRN/Life Technologies genotyping method [37]. Furthermore, SATuRN members can access the reagents at a discounted price.

Clinical Setting

The described protocol has been implemented in the monitoring and surveillance of drug resistance in a rural community in KwaZulu-Natal. A total of 604 genotypes were generated from clinical samples between December 2010 and May 2013 at an amplification rate of 95% for samples with viral loads >1,000 RNA copies/ml. This clinical HIV drug resistance study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (ref. BF052/10) and the Health Research Committee of the KwaZulu-Natal Department of Health (ref. HRKM 176/10). Individual patient reports were generated and sent back to the clinics for patient management.

Seventy two (72) genotypes were also generated as part of a surveillance of transmitted drug resistance study, nested within a large prospective population-based HIV surveillance study. The primary samples were needle prick whole blood collected in EDTA microtubes. At genotyping there was an amplification rate of 79%[19]. Ethics approval for the genotyping of samples from the surveillance study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (ref. BE066107).

Table and Figure Legends

Table 1. Reverse transcription, PCR and sequencing custom primers used in the generation of a 1197 bp pol fragment covering all the 99 HIV-1 Protease codons and the first 300 codons of the reverse trascriptase gene.

Table 2. dNTP/Primer mix for the reverse transcription reaction.

Table 3. Enzyme mix for the reverse transcription reaction.

Table 4. Master mix for the nested PCR.

Table 5. Master mix for the sequencing reactions.

Table 6. Comparative results from a parallel anaylsis between the Viroseq genotyping method and the in-house method using a panel of samples provided by the ANRS.

Figure 1. Nested PCR cycling conditions.

Figure 2. Gel confirmation of PCR amplification using 1% agarose gel electrophoresis and a 200 bp ladder.

Figure 3. Scheme representation of a 96 Well Plate with 12 patient samples being sequenced with 4 primers each (RTC1F, RTC2R, RTC3F and RTC4R).

Figure 4. PCR cycling conditions for sequencing.

Figure 5. Use of a HKY Neighbor Joining tree done as part of sequence quality assurance. There are four pairs/clusters of sequence with very short genetic distances. The genetic distance between RES655 and RES655_1 (same samples sequenced on different days) is 0.003. The is a potential error with the RES637_1/RES638 pair as their genetic distance is too short (0.075) for samples from different epidemiologically unlinked individuals. There is another RES637 on the tree with a distance of 0.075 when compared to RES638_1. The CQ01/CQ02 cluster suggests that the two samples from the panel are duplicates of the same sample. They cluster together with the subtype B reference sequence confirming the subtype assigned by the REGA Subtyping tool. CQ05 and CQ04 clustered with subtypes A and G respectively, whereas the REGA subtyping tool classified them as A and CRF02_AG respectively. Another useful tool for HIV subtyping and recombination is SCUEL, which is available at http://www.datamonkey.org

Figure 6. Comparison of contiguous sequences from six vs four sequencing primers for the generation of the 1197 bp pol sequence covering all 99 HIV-1 protease codons and the first 300 codons of the reverse transcriptase gene.

Discussion

Several low cost in-house methods have been described in efforts to try to make HIV drug resistance genotyping more affordable[33,34,36]. There is no doubt of the need to integrate drug resistance testing into the continuum of care for individuals on antiretroviral therapy in resource-limited settings. However, most of the reported methods focus on the application of drug resistance genotyping in the surveillance of drug resistance at a population level. The SATuRN/Life Technologies genotyping method is a fully integrated protocol for surveillance and monitoring of drug resistance. This method was designed to be an affordable protocol implementing mostly open source and open access bionformatics resources for the interpretation of drug resistance and generation of reports for clinical management.

It was shown through comparison with the FDA approved Viroseq genotyping method to be accurate in identifying drug resistance mutations from a panel of ANRS proficiency testing samples, in 100% of laboratory panel samples that were successfully amplified. The accuracy was also assessed on clinical samples of subtype C viruses, the most dominant subtype in

southern Africa. The method was as accurate on subtype C samples as it was on subtype A and B. However, if the method would be used in other parts of the world where CRF02_AG is prevalent, there is a need for the modification of the primers since the method failed to amplify one of the panel samples that was shown to have CRF02_AG. Alternatively, a degenerate set of primers sensitive to all group M viruses[33,36] could be used in regions where the subtype distribution is more heterogeneous[38].

The sensitivity of the reverse transcription and PCR can be increased by extracting RNA from higher volumes of plasma, such as 500 µl. The plasma can be centrifuged at 21,000 x g for 90 min to concentrate the viral particles before proceeding with the protocol as described by the QIAamp viral RNA extraction mini kit.

As shown, the new method has an additional advantage that it produces comprehensive reports for individual patient management. These reports are a consolidation of the genotype, the immunological and virologic monitoring data as well as clinical and treatment history from RegaDB. This is accompanied by a detailed laboratory interpretation of the resistance profile followed by an equally detailed review of the patient's clinical history as well as treatment recommendations. The use of a specialist physicians to review the reports and provide treatment recommendations for the patients provides the much-needed mentorship for nurse practitioners as well as inexperienced clinicians, who are increasingly providing ART in South Africa as part of the WHO recommendations for task shifting. These clinical reports have been shown to be effective teaching aids for clinicians with little or no experience in drug resistance management. From a patient perspective, our method reduces the need to travel to centralised sites to access specialist HIV services.

Thus, the described protocol taken as a whole provides a good platform through which HIV drug resistance management can be integrated, at an affordable cost, into the continuum of care for HIV infected individuals failing ART. The data generated can be used for epidemiological purposes to assess the evolution and transmission of drug resistance in the community. The size of the pol fragment generated is good enough for more complex phylogenetic analysis which will produce better understanding of the epidemic at population level.

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*Figure
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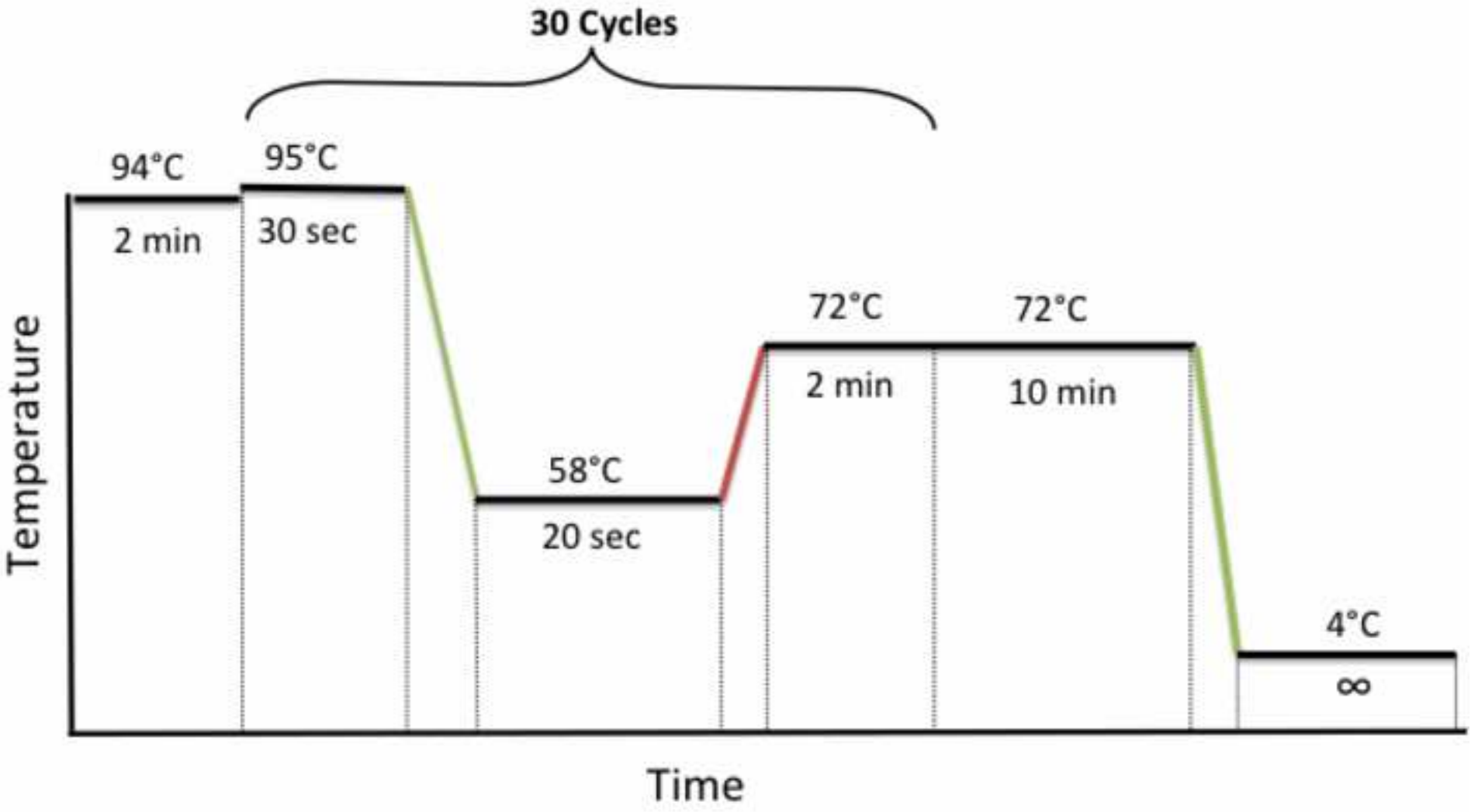

*Figure

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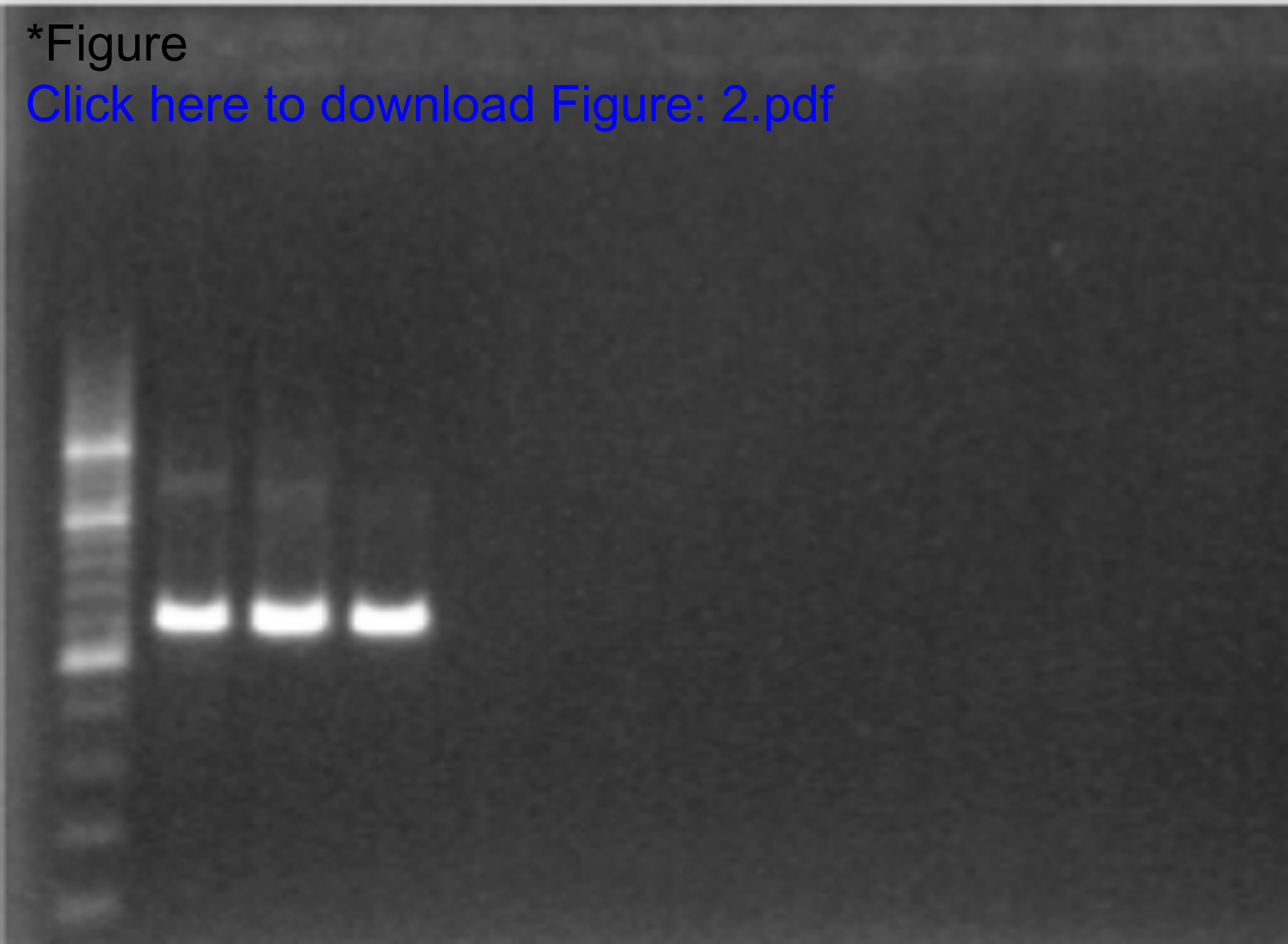

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	1	2	3	4	5	6	7	8	9	10	11	12
A	1_RTC1F	3_RTC1F	5_RTC1F	7_RTC1F	9_RTC1F	11_RTC1F						
B	1_RTC2R	3_RTC2R	5_RTC2R	7_RTC2R	9_RTC2R	11_RTC2R						
C	1_RTC3F	3_RTC3F	5_RTC3F	7_RTC3F	9_RTC3F	11_RTC3F						
D	1_RTC4R	3_RTC4R	5_RTC4R	7_RTC4R	9_RTC4R	12_RTC4R						
E	2_RTC1F	4_RTC1F	6_RTC1F	8_RTC1F	10_RTC1F	12_RTC1F						
F	2_RTC2R	4_RTC2R	6_RTC2R	8_RTC2R	10_RTC2R	12_RTC2R						
G	2_RTC3F	4_RTC3F	6_RTC3F	8_RTC3F	10_RTC3F	12_RTC3F						
H	2_RTC4R	4_RTC4R	6_RTC4R	8_RTC4R	10_RTC4R	12_RTC4R						

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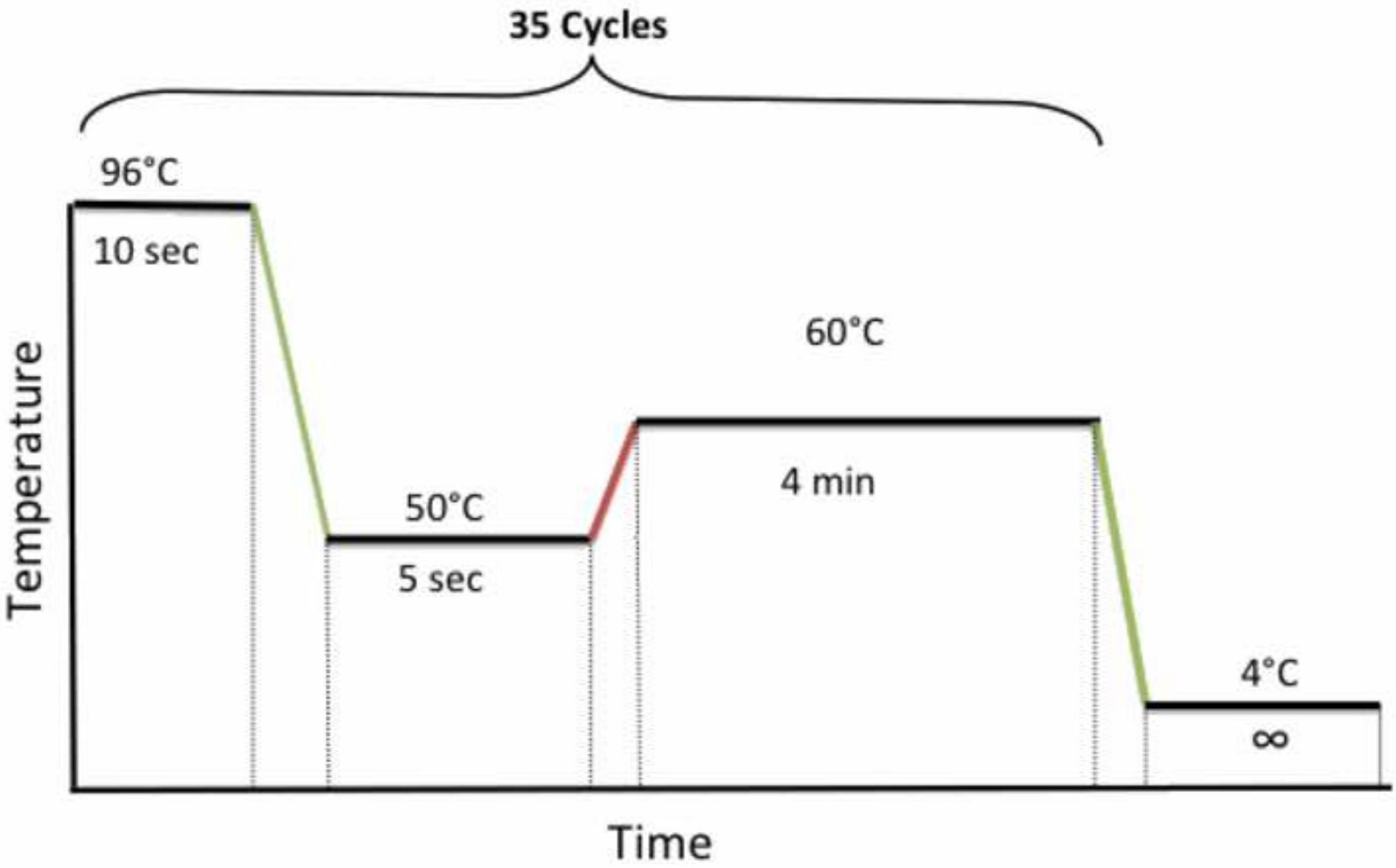

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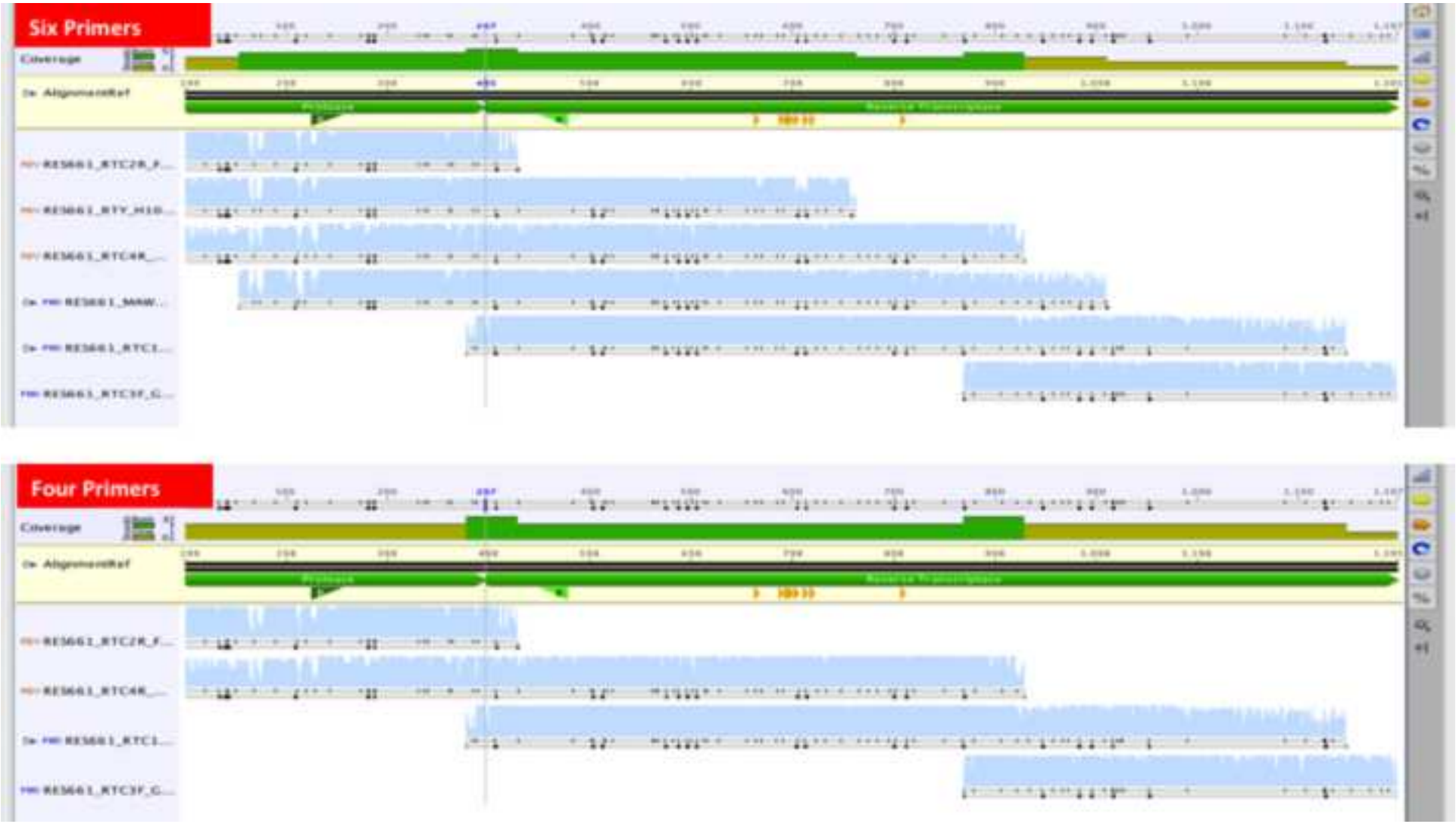

Table
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Primer Name	Sequence	Length	Direction
MAW-26	TTGGAA ATGTGGAAAGGAAGGAC	23	Forward
RT-21	CTGTATTTCAGCTATCAAGTCCTTTGATGGG	31	Reverse
Pro-1	TAGAGCCAACAGCCCCACCA	20	Forward
RT-20	CTGCCAATTCTAATTCTGCTTC	22	Reverse
RTC1F	ACCTACACCTGTCAACATAATTG	23	Forward
RTC2R	TGTCAATGGCCATTGTTTAACCTTTGG	27	Reverse
RTC3F	CACCAGGGATTAGATATCAATATAATGTGC	30	Forward
RTC4R	CTAAATCAGATCCTACATACAAGTCATCC	29	Reverse
RT-y	GTGTCTCATTGTTTATACTAGG	22	Reverse
MAW-46	TCCCTCAGATCACTCTTTGGCAACGAC	27	Forward

HXB2 Position	
2028-2050	**1st round PCR**
3539-3509	**1st round PCR**
2147-2166	**2nd round PCR**
3462-3441	**2nd round PCR**
2486-2508	**Sequencing**
2630-2604	**Sequencing**
2956-2994	**Sequencing**
3129-3101	**Sequencing**
2967-2946	**Sequencing**
2251-2277	**Sequencing**

Table
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Reagent	Volume (㎖) / reaction	Concentration/manuscripts
RT21 (5pmol/ml)	0.5	0.2
dNTP (10 mM)	0.5	0.4
Total	1	

Table
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Reagent	Volume (ml) / reaction	Concentration/reaction
First Strand Buffer (10 x)	1	1
$MgCl_2$ (25 mM)	2	4
DTT (0.1M)	1	0.008
Rnase OUT (40U/ ml)	0.5	16
Superscript III Reverse Transcriptase (200U/ ml)	0.5	8
Total	5	

Table
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Reagent	Volume (㎖) / reaction
DEPC treated water	18.4
PCR Buffer (10 x)	2.5
$MgCl_2$ (50 mM)	1
dNTP mix (10 mM)	0.5
Froward primer (5pmol/㎖)	0.25
Reverse primer (5pmol/㎖)	0.25
Platinum Taq Polymerase (5U/㎖)	0.1
Sub-total	23

Final Concentration/Reaction
-
1
2
0.2
0.05
0.05
0.02
-

Table
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Reagent	Volume (㎖) / reaction	Concentration/reaction
DEPC treated water	6.1	
Sequencing Buffer (5 x)	2	1
Primer (3.2pmol/㎖)	0.5	0.16
Big Dye terminator Sequencing mix	0.4	-
Total	9	

Table
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	Viroseq				
Sample I.D	**Subtype**	**Quality score**	**PR Mutations**	**RT mutations**	**Subtype**
CQ01	B	99.9	M46L, I54L, V82A, L90M	D67N, T69D, K70R, M184V, T215V, K219Q	B
CQ02	B	99.5	M46L, I54L, V82A, L90M	D67N, T69D, K70R, M184V, T215V, K219Q	B
CQ03	NA	NA			NA
CQ04	CRF02_AG	98.4	I54V, V82F, I84V	M41L, L74I, L210W, T215Y, V108I, Y181C	NA
CQ05	A	99.7		K103N	A

Inhouse			% NA Similarity
Quality score	**PR mutations**	**RT Mutations**	
99.2	M46L, I54L, V82A, L90M	D67N, T69D, K70R, M184V, T215V, K219Q	100
99.5	M46L, I54L, V82A, L90M	D67N, T69D, K70R, M184V, T215V, K219Q	100
NA		NA	NA
NA	NA	NA	NA
93		K103N	100

*Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Qiamp Viral RNA mini kit	Qiagen	BC52906	RNA Extraction
Superscript III 1st strand Synthesis kit	Life Technologies	18080051	Reverse Transcription
SATURN/LiFE Technologies Custom Primers	Life Technologies	4473517	PCR
Platinum Taq	Life Technologies	10966026	PCR
PureLink QUICK PCR Purification Kit	Life Technologies	K310002	PCR
Viroseq	ABBOTT	4J94-20	Reverse Transcription and PCR
Agarose Tablets (Dnase/Rnase free)	BIOLINE	BIO-41027	PCR
TBE Buffer	MERCK	1.06177.2500	PCR
O'Range Ruler 200bp DNA Ladder	Fermentas	FE SM0633	PCR
Novel Juice	GeneDireX	LD001-1000	PCR
MiniBis Bioimaging System	DNR Bioimaging Systems Ltd		Gel Documentation
Power Pac 300	BIORAD		Gel Electrophoresis
Big Dye Terminator Kit ver 3.1	Life Technologies	4337456	Sequencing
Arrays	Life Technolgies	4319899	Sequencing
PoP	Life technologies	4363785	Sequencing
10 x EDTA Buffer	Life Technologies	402824	Sequencing
Formamide	Life Technologies	4311320	Sequencing
5 x Sequencing Buffer	Life Tecgnologies	4336697	Sequencing
3130 xl Genetic Analyzer	Life Technologies		Sequencing
GeneAmp PCR System 9700	Life Technologies		RT/PCR/Sequencing
Centrifuge 5804	EPPENDORF		Sample Processing
Centrifuge 5415R	EPPENDORF		RNA Extraction
Centrifuge 5415R	EPPENDORF		RT and PCR
Centrifuge 5415D	EPPENDORF		PCR Product Clean up
Centrifuge 5810	EPPENDORF		Sequencing Clean up
Picofuge	BIORAD	C1301-230V	RT and PCR
Vortex Genius 3	IKA		RNA Extraction
Vortex Genius 3	IKA		reagent preparation
Vortex mixer	IKA		Sequencing Clean up
NanoDrop 2000 UV/VIS spectrophotometer	ThermoScientific		DNA quantification
3M Sodium Acetate	MERCK	567422	Sequencing Clean up
Absolute Ethanol	MERCK	SAAR2233540LP	Sequencing Clean up
1.5ml SARSTEDT Tubes	BIODEX	72.692.005	RNA Extraction
2ml SARSTEDT Tubes	BIODEX	72.693.005	RNA Extraction
2ml Collection tubes	SCIENTIFIC GROUP	MCT-200-NC/S	RNA Extraction
Optical MicroAmp 96 Well reaction plates	Life Technologies	N8010560	Sequencing
200ul 8 Strip StarPCR Tubes with attached flat caps	STAR Lab - supplied by CELTIC	A1402-3700	RT and PCR
200ul PCR individual tubes	Scientific Group	CR/3745	RT and PCR
Geneious	Biomatters		Sequence analysis
Internet Access			Preferrably high speed
Web resources			
hivdb.stanford.edu	Stanford University		Drug reistance analysis

http://bioafrica.mrc.ac.za:8080/regadb-ui/RegaDB	SATuRN		database
http://bioafrica.mrc.ac.za/tools/pppweb.html	SATuRN		Sequence quality tool

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Author(s): **Justen Manasa**

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Institution:	**University of KwaZulu-Natal**
Article Title:	**An Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings.**
Signature:	 Date: **31 May 2013**

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Dear Mr. Manasa,

Your manuscript JoVE51242 'An Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings.' has been peer-reviewed and the following comments need to be addressed.

Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

Often reviewers request the addition of a large amount of details or explanations. We realize that, especially in the protocol section, brevity and clarity are important for a JoVE publication and expect the focus to be on providing a framework for the method presented rather than a comprehensive review of the research field. Please address each comment in your rebuttal and note if you choose not to include the requested information in the text and the reasoning behind this decision.

Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.

Editorial Answers:
We would like to thank the editor and reviewers for the useful and positive comments. All of comments on the video and manuscript have been addressed and we trust that the paper and videos are ready for publication.

In certain instances (for example, question 1 and 2 of first reviewer), we followed the editorial suggestion to keep the paper succinct and did not edit the manuscript but provide a detailed answer to the reviewer.

Editorial comments:

Audio issues
* 0:44 - The audio levels increase significantly here. Either the audio levels before this point should be raised, or the audio levels after it should be lowered.
* 12:48 - There is some stray audio here that should be removed.
* 16:00 - There is some stray audio here that should be removed.

Formatting and text issues
*On-screen text and graphics at the following time points are too small to be seen clearly when the video is scaled to our webplayer's size. We recommend the authors view their submission on the JoVE site and then adjust the text and graphics accordingly:

-0:01, 19:56 - Authors' affiliations
-1:13 - Text in the tables
-2:04, 7:48, 8:48 - The tables would benefit from being increased in size
-11:18 - 18:38 - Specific actions in the software are being described in the voice-over, but cannot be seen clearly in the video when viewed on our website. The authors will need to zoom in on areas of interest in order to allow the viewer to see any detail.

Response: The video was edited to address the above concerns.

Reviewers' comments:

Reviewer #1:
Manuscript Number: JoVE51242
The authors have provided a description of an "Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings". The development of alternative HIV-1 drug resistance genotyping assays has become popular over the years, initially due to suboptimal sensitivity of commercial assays for non-B subtypes, but also to overcome high costs of commercial assays. Manasa J. et al. concluded that, the in-house method was cost effective and produced similar results to those of ViroSeq method (commercial US FDA approved).

Major concerns:

1. **Concern:** The generalized heterosexual epidemics in Africa and Asia have expanded and diversified to include nine major HIV-1 subtypes (A-D, F-H, J and K) and mosaic circulating recombinant forms (e.g. CRF01_AE and CRF02_AG of the 51 CRFs) [Lihana R et al. AIDS Rev 2012; Hemelaar J et al. AIDS 2011]. Migration and globalization has contributed to the spread of non-B subtypes contributing to 20-60% of new infections in Europe, Asia and America [Tebit M et al. TLID 2011]. The described assay cannot serve as an alternative to commercial assays for HIV-1 drug resistance genotyping in routine diagnostics, and for surveillance and monitoring of drug resistance in resource-limited settings (RLS). A group-M subtype-independent genotyping assay, using universal primers for detection of HIV-1 drug resistance is highly desirable. The method described here failed to amplify CRF02_AG a predominant HIV-1 strain co-circulating in West and Central Africa.
   Response: We appreciate the reviewer concern on the need of a method using universal primers. The method's inability to amplify CRF02_AG was clearly noted as a limitation in the discussion (page 23). We are currently working on the modification of the current primers in order to enable them cover more subtypes. However, for this current manuscript primer issues were not of main importance as the objective of this manuscript is to provide a framework for the method presented rather than a comprehensive list of primers and a review of the research field. Readers can plug in different primers into the genotyping system described, which covers reverse transcription, PCR, Sequencing and bioinformatics, without the need to

change the protocol presented in this manuscript. As part of the Southern African Treatment Resistance Network (SATuRN – http://www.bioafrica.net/saturn/) and the PharmAccess African Studies to Evaluate Resistance (PASER – http://www.pharmaccess.org/Default.asp?Page=126), the two largest HIV drug resistance network in Africa, we are in the process of developing a section of the website in bioafrica.net that present different primers used for HIV drug resistance genotyping in Africa and a question and answer section that can be used for trouble-shooting similar in-house and affordable genotyping protocols. We trust that this web-resource will be a more appropriate area to present detail on primer issues and subtype distributions than this manuscript and video.

2. Concern: The running cost per test for both the ViroSeq ($300) and the in-house methods has not been evaluated (in terms of affordability). The authors claim that this method was designed to be an affordable protocol implementing mostly open access and open access bioinformatics resources for the interpretation of HIV drug resistance.

   Response: Overhead costs significantly affect the running cost of diagnostic tests and their impact in different settings varies. That is the reason why we only highlighted the stages associated with significant cost reductions in the described protocol instead of actual amounts. The costing is also significantly affected by exchange rates as the reagents are imported. As of October 2013 the reagents cost plus a 10% charge for overhead cost (other consumables not included in the described package and maintenance of equipment) for the SATuRN genotyping protocol was approximately R900 whereas that of Viroseq was approximately R2100.

3. Clarity of the procedures (completeness of required information, instructions and wording) will be of interest to scientists in other institutions that wish to apply the same or similar techniques.

   a. **Concern:** Page 7 - It will be helpful to the reader if the authors provide brand name, manufacturer for RNA extraction kit. It is hard to determine if this is the QIAamp Mini Kit (see page 21, 1st paragraph).
      Response: The name of the RNA extraction kit is provided on the materials sheet provided. “Qiagen” was changed to “QIAamp” on page 21
   b. **Concern:** Page 11 - PCR Product clean up: DNA quantification is often recommended after 8.12 to determine concentration, purity (ratios of optical densities at 260nm and 280nm) and yield. This will be required in section 9.8 - Sequencing reactions on page 12. Determination of optical densities at 260nm and 280nm: o Visually check that the DNA is completely dissolved (Although viscous, the solutions should look homogeneous with no large "globs" of partially dissolved DNA) or Use 1.0 to 1.5 <mu>l undiluted DNA to read

concentration on Nanodrop apparatus or Calculate the concentration of the stock solution of DNA in mg/ml (Recall that 50<mu>g/ml gives an optical density at 260nm of 1 unit.); also calculate the ratio of absorbance at 260 to 280nm [Ratio should be ~ 1.8; very low ratio (~ 1.6) may indicate significant protein contamination and very high ratio (~ 2.0) may indicate significant RNA contamination.
Response: Added the DNA concentration measurement step 8.12 after the PCR product clean up

4. Concern: Page 15 - Sequence Quality Assessment (HIVDB): There are 2 separate tools on the website Calibrated Population Resistance (CPR) tool. This analysis designed to evaluate sequences from treatment-naïve subjects for assessment of the prevalence of transmitted drug resistant HIV. It uses a list of mutations (the SDRM list) to categorize viruses as having or lacking evidence of ARV drug selection pressure. HIVdb resistance analysis program. This program provides a drug resistance/susceptibility assessment using a mutation scoring system and 5 levels of predicted susceptibility.
Response: Page 15, section 11.2.1 mentions "**HIVDB program**". The first two segments of the report generated 1) Summary data, 2) Sequence Quality Assessment provide provides the sequence quality information in addition to the mentioned resistance/susceptibility assessment mentioned above.

Minor concerns:

5. **Concern:** Page 28 - Figure 2 should be reported in the text and a legend provided.
Response: The place where Figure 2 should be added is indicated on Page 10, section 7.2.

6. **Concern:** Page 28 - Figure 5, Subtype assigned by the REGA Subtyping tool. Any explanation on the cluster of transmission (Phylogenetic tree)?
Response: The phylogenetic tree in this manuscript is used as a quality monitoring too as indicated in the figure legend. It was not used to infer the subtypes of the samples.

7. **Concern:** Subtype Classification Using Evolutionary Algorithms (SCUEAL) procedure, freely available tool accessible on the Internet is the one of the most reliable tools for HIV pol subtyping.
Response: We appreciate the reviewer's preference in terms of the subtyping method. However the method we opted for is also considered to be one of the best and has more than 280 citations in peer-reviewed journals. However, we have added to figure 5 legend mention of the usefulness of SCUEL "Another useful tool for HIV subtyping and recombination is SCUEL, which is available at http://www.datamonkey.org"

References (SCUEAL):

1. Kosakovsky Pond SL, Posada D, Stawiski E, Chappey C, Poon AFY, et al. (2009) An Evolutionary Model-Based Algorithm for Accurate Phylogenetic. Breakpoint Mapping

and Subtype Prediction in HIV-1. PLoS Comput Biol 5(11): e1000581. doi:10.1371/journal.pcbi.1000581

Reviewer #2:

Manuscript Summary:

The paper describes comprehensively an affordable HIV drug resistance testing method for surveillance and patient monitoring for resource limited settings. The paper diligently outlines various aspects of a lengthy process, namely; processing of blood specimens, RNA extraction, reverse transcription, PCR, gel electrophoresis, sequencing and bioinformatics. The paper forms a valuable resource for both novices and experienced scientist in the field of HIV drug resistance genotyping and interpretation of the genotypes. The rationale of the development of the method is clearly laid out and its limitations clearly stated. There is no doubt that the method will evolve to deal with these limitations as it is widely adopted and modified in various institutions.

Major Concerns:

No major concerns

Minor Concerns:

Concern: I believe the use of 4 primers and reduced volume of reagents definitely results in cost savings. Without any figures attached to show as an example it may appear less convincing that indeed the adoption of the method would cut the costs. However, I strongly feel this will not hamper the adoption of the method.

Response: Overhead costs significantly affect the running cost of diagnostic tests and their impact in different settings varies. That is the reason why we only highlighted the stages associated with significant cost reductions in the described protocol instead of actual amounts. The costing is also significantly affected by exchange rates as the reagents are imported. As of October 2013 the reagents cost plus a 10% charge for overhead cost (other consumables not included in the described package and maintenance of equipment) for the SATuRN genotyping protocol was approximately R900 whereas that of Viroseq was approximately R2100.

Additional Comments to Authors:
Please note that these are only suggestions:

Suggestion: Page 5 paragraph 3
The South African HIV treatment and monitoring guidelines do did not currently recommend the use of GRT in guiding choice of ART for individuals failing first-line or second-line regimens17. Individuals are were switched based primarily

Response: **Revised the statement to read as follows;**
"The South African HIV treatment and monitoring guidelines **do** not currently recommend the use of GRT in guiding choice of ART for individuals failing first-line "

Also added the following statement at the beginning of page 6;

"Already, in the 2013 South African treatment guidelines there is now recommendation of GRT at time of second-line failure for adults and at time of first- or second-line PI-based regimen failure for children"

Suggestion: Page 6 paragraph 1
Please explain cost-neutral

Response: Cost neutral was explained on page 6 as follows;

"Considering the cost of the second line regimen drugs which are relatively more expensive that the first line drugs, using GRT to identify patient who truly need to be switched to second line therapy will not result any additional cost to the program"

Suggestion: 1 Ethylenediaminetetraacetic acid (EDTA) Whole Blood Processing
1.1 Working in a biosafety cabinet, allow the EDTA whole blood sample to reach room temperature [where was the blood stored].

Response: Added the storage conditions for the blood before storage;

"Blood can be processed immediately after collection of can be stored at 4°C for no more than 24 hours"

Suggestion: 1.2 For each sample, label three enough cryovials for storage of plasma with the sample identification (ID), storage material (plasma) and date.

Response: **The statement was changed to:**

"For each sample, label enough cryovials with the sample identification (ID), storage material (plasma) and date"

Suggestion: RNA Extraction
2.1 Prepare an extraction worksheet with the IDs of the samples to be extracted including positive and negative plasma controls [Include example of sample worksheet].

Response: A sample of the work worksheet was added as additional information.

Suggestions: 2.3 Working in the Bio-Safety Cabinet, add 200<mu>l sample to the corresponding 2ml microcentrifuge tube of working lysis solution.

Response: The word “microcentrifuge” was added to 2.3 on page 7

Suggestion: 2.9 Transfer column to a new collection tube and discard the old collection tube containing the filtrate. Repeat the above step 2.8 (above) two more times.

Response: The word “above” was added to 2.9 on page 8

Suggestion: 2.12 Add 500 <mu>l wash buffer AW2 and centrifuge for at 20, 000 x g for 3 min. Repeat step 2.11.
Response: “Repeat 2.11(above)” was added to 2.12 on page 8

Suggestion: 2.20 If testing is to be performed immediately, store at 4°C [Please indicate how long can stay at 4oC without degradation]. However, if testing is to be delayed then place at -80°C immediately. NB: do not freeze/thaw the samples more than 3 times.

Response: We do not keep RNA at 4°C for no more than 6 hours. This information was added to section 2.20.

Suggestion: 3 Reagent Preparation for Reverse Transcription

3.1 Before starting, calculate the volumes of each of the reagents required for the number of samples being processed including, the positive and negative plasma controls. Also add a reagent control.

3.2 Prepare the deoxyribonucleotide triphosphate (dNTP)-primer mix by adding 0.5 <mu>l of the reverse primer RT21 and 0.5 <mu>l of the dNTP mix to a clean, sterile 200 <mu>l PCR tube followed by briefly pulse vortexing, see Table 3.

3.3 Aliquot 1.0 <mu>l of the dNTP-primer mix to 200 <mu>l PCR tubes.

[Please note that the above instructions need revision. 3.2 must indicate that to make the volumes calculated in 3.1, the reagents

must be mixed in proportions as stated in 3.2 for example if one needs 20 µl mix, one would add, 10 <mu>l of the reverse primer RT21 and 10 <mu>l of the dNTP mix to a clean]. Then one can go to 3.3.

Response: **3.2 was edited to read as** "Using the calculated volumes from 3.1 (above), prepare the deoxyribonucleotide triphosphate (dNTP)-primer mix in a clean, sterile 200 µl PCR tube followed by briefly pulse vortexing. Each sample should have 0.5 µl of the reverse primer RT21 and 0.5 µl of the dNTP, **see Table 3**"

Suggestion: 4 Reverse Transcription

4.2 After the addition of the RNA, move to the PCR room with both dNTP/primer/RNA mix tube and RT Enzyme mix tube on a cold block or ice.

Response: 4.2 was edited to read as "After the addition of the RNA, move to the PCR room with both dNTP/primer/RNA mix and RT Enzyme mix tubes on a cold block or ice".

Suggestion: 4.3 Briefly centrifuge the dNTP/primer/RNA mix tubes (from step 4.2) and place them into a thermocycler.

Response: 4.3 was edited to read as "Briefly centrifuge the dNTP/primer/RNA mix tubes (from step 4.2) and place them into a thermocycler".

Suggestion: 4.13 The complementary DNA (cDNA) can be used immediately or can be stored at - 20oC or colder until needed [Please mention something about long term storage].

Response: The sentence, "However, the long term storage of cDNA should be at -80°C". was added to section 4.13 on page 10.

Suggestion: 5 Reagent Preparation for PCR

5.2 Add the water, 10 X buffer, MgCl2, dNTPs, and primers in the amounts shown on Table 5 and vortex [for consistency please include the volumes alongside the reagents].

Response; The volumes were included as suggested on section 5.2 on page 11.

Suggestion: 6 Nested PCR

6.2 Close the tubes, put the samples in the thermocycler and run the PCR programme shown on Figure 1. (Place Figure 1 here) [describe the cycles in words before referring to th diagram].

Response: The cycles were described in section 6.2 page 11.

Suggestion: 7 Gel Electrophoresis

7.1 Set up and run 1.0% Agarose gel electrophoresis at 100V and 400W for 40 min to evaluate the PCR amplification. [Please include a section on gel preparation including the addition of fluorescent dye such as ethidium bromide or whichever one. Include a bit of section on the loading buffer and inclusion of molecular weight marker].

Response: Section 7 on page 12 was expanded to provide more detail on Gel preparation and loading.

Suggestion: 7.2 Positive amplification can be visualized under UV light as 1315 bp f ragment, Figure 2. (Place Figure 2. here)

Response: "under UV light" was added to section 7.2, which is now 7.2.5

Suggestion: 8 PCR Product clean up

8.1 In preparation for the sequencing reaction, the positive second round PCR products are cleaned up using the PureLink PCR purification kit [manufacturer].

Response: The name of the manufacturer was not included in section 8.1 to be consistent with other sections where different reagents were mentioned. However the name of manufacture "Life Technology" is on the materials table.

Suggestion; 11.3 Sequencing Quality Control

11.3.3 If no problems are identified, re-sequence both the old and new samples [not clear].

Response: 11.3.3 was rephrased to, "If no problems are identified, repeat the analysis of both the old and new samples from the RNA extraction stage".

Suggestion: 11.4 Phylogenetic Analysis

11.4.3 construct a phylogenetic tree using PHYML, Geneious tree builder or other tree builders in Geneious.

Response; The omitted word "using" was added to section 11.4.3