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](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.

1. 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.

1. 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.
   1. **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

* 1. **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

1. 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:

1. **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.

1. **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.

1. **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