**Rebuttal letter for submitted manuscript JoVE58715 “Determining 3’-termini and sequences of nascent single stranded viral DNA molecules during HIV-1 reverse transcription in infected cells” by Pollpeter et al.**

**We thank the editor and reviewers for their careful reading of our manuscript. We have made all the requested changes and believe this improved the manuscript for the reader. Please find the actions taken in bold and italics.**

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. **- *Done***

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].” **– *See submitted document, lease advice if any additional information is required.***

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. – ***Changed where applicable***

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: OptiMEM, Alliance, Qiagen, Cutsmart, LoBind, Eppendorf, etc. **- *Done***

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **- *Done***

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion. **– *Done. This is the main change in the resubmission. Several notes were moved to the discussion, other, shorter notes were incorporated. Some steps were split into sub steps and hence step numbering has now changed.***

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **- *Done***

Some examples:

1.1.2: Please specify the incubation temperature. **- *Done***

1.1.3: What happens after replacing with new medium? Is the cultured incubated? Please specify. **- *Done***

1.1.4: What happens after filtration? **- *Done***

1.1.5: Please specify ultracentrifugation parameters (force and time). What container is used in this step? Please break up into sub-steps. **- *Force and time was already indicated, changed others.***

1.2.3: Please specify the incubation conditions. Please specify throughout. **- *Done***

8. Please ensure that conditions and primers are listed all PCR procedures. **– *Double checked, though not sure where any were missing.***

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion. **– *Done, see comment 6 above.***

10. References: Please do not abbreviate journal titles. ***– Changed to full journal titles.***

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

This manuscript describing the method to determine 3'-termini and sequences of HIV viral DNA in infected cells will significantly help researchers in the field. The manuscript is very well written, detailed and very comprehensive. Being a HIV researcher myself ( although not specialized in the RT step), I had no difficulty whatsoever in understanding this protocol. I strongly recommend this for further consideration.

Major Concerns:

None

Minor Concerns:

None

**Reviewer #2:**

Manuscript Summary:

The method addresses a gap in research enabling detailed analysis of reverse transcripts in HIV infected cells. Previously, methods would detect if reverse transcription had reached a specific stage or not, but were unable to determine the sequence of the reverse transcripts and were based on population level analyses. This method enables capture and sequencing of individual nascent reverse transcribed (-) DNA molecules. There is also a method for analyzing sequences to determine the length of the transcript and deamination events (if APOBEC3 enzymes are being studied).

Major Concerns:

The analysis of sequences is the major culmination of a very involved method. However, the reference to the website alone does not seem to be sufficient; especially if a researcher has not done their own sequence analysis previously. Some more detail of how to use the GitHub and SeqParse within the manuscript is warranted. ***– We have now added a step-by-step description of how to install, set up and run our in-house script, see section 8.***

Minor Concerns:

Section 1.1.4: It is not clear if the 48 h later is after the media change or the transfection. ***– We have now specified that it is after the transfection***.

Section 1.1.5: It is not clear that the virus will be in a pellet below both the sucrose and supernatant, needs to be directly stated. ***- Done***

Section 4.2.5: It is not clear until later in the protocol that each high, med, and low gel fragment should be kept separate. ***– We have now made this clear at several steps (see step 4.2.1 and in a note after step 4.2.6)***

Figure 2a: Has an uncompiled reference. ***- Changed***

Representative Results Section: When discussing the oligos and possibly having incomplete products; what was the purity of the oligos that were ordered (Desalted, HPLC, gel purified?); would your recommend purification to avoid this or it still occurs? ***– We have now added the information that we had ordered oligos as HPLC purified. We don’t know, whether further purification would avoid the presence of incomplete products altogether.***

**Reviewer #3:**

The manuscript entitled 'Determining 3'-termini and sequences of nascent single stranded viral DNA molecules during HIV-1 reverse transcription in infected cells' by Pollpeter et al. describes a detailed protocol to analyze nucleotide sequences of the early reverse-transcribed DNA products in HIV-1-infected cells. The authors' group recently reported a possible molecular mechanism of APOBEC3G-mediated reverse transcription inhibition (ref #6), which was conducted by using the protocol. To date, many retrovirus researchers have attempted to determine the 3'-termini of the early reverse-transcribed DNA intermediates in HIV-1-infected cells. But there were no feasible and sensitive assay systems to analyze them, because of some technical difficulties. In this manuscript, the authors optimized the best method of the adaptor ligation to prepare a DNA library for the next generation sequencing tool, Illumina MiSeq and elaborated this protocol. Overall, this manuscript is well written, important and very informative. I have only a few minor comments as described below.

Minor comments:

1: line 85. A typo. The "RML-RACE" should be "RLM-RACE". ***- Changed***

2: lines 191-207. Specify the temperature(s) in these processes. ***– Added, all at room temperature.***

3: lines 239 and 245. Should 40% PEG be used here? In the ref #6 that the authors previously reported, it says 50% PEG. ***– We have used both 50% PEG and 40% and did not find a difference. We currently use 40%, simply because the 50% product was discontinued from out chosen company***.

4: lines 542. The "long (116-134 nt)" is correct? It seems "long (116-120)". – ***134 is indeed incorrect, we have now changed this to 120.***