**Rebuttal letter**

**Ref: JoVE58572\_R1**

**Title: CRISPR/Cas9-based genome engineering to generate Jurkat reporter models for HIV1**

**infection with selected proviral integration sites**

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We would like to thank the editors for the thorough review of our manuscript. We have addressed the comments as follows:

Comment [A1]: We enforce a 10-page limit on the protocol section. The current protocol section is close to 11.5 pages long. Can some portions be trimmed or moved to a supplementary file?

* We would suggest to move one part of step ‘3.4 Southern blot Analysis for verification of targeting in selected clones’ to a supplementary file. In step 3.4 to 3.4.7 the purpose of Southern blot analysis, design of Southern probes and generation of Southern probes and preparation of gDNA from targeted single cell clones is explained. We would keep this part in the main protocol. The later steps (3.4.8 – 3.4.18) are more general, and we have moved them to a supplementary protocol.
* Please find the supplementary protocol with step 3.4.8 to 3.4.18 of the Southern blot protocol uploaded together with the main manuscript. We have added an explanatory note in line 541 to 548 of the revised manuscript which refers to the supplementary protocol.

Comment [A2]: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. Please address the comments below.

The protocol has been revised carefully and more details have been added, especially to step 1 and following. All comments have been addressed.

Comment [A3]: Some stes were adjusted for language and clarity. Steps that were not in the imperative voice were made into notes. Notes are generally not filmable and have been excluded from highlighting.

We would like to thank the editors for the thorough review of our protocol.

Comment [A4]: Please provide additional details for inputs you enter in the „organim“, „target reion“, etc fields.

Detailed steps for selection of gRNAs using E-CRISP webtool have been added in step 1.1.2.1 to 1.1.2.4.

Comment [A5]: Please mention all button clicks and field entries in order to esure filmability.

Single actions for blasting of gRNA sequences have been added in step 1.1.3.1.

Comment [A6]: Please add this to the reference list and use superscripted citation numbering.

Protocol for gRNA cloning in pX330 has been added as reference #12.

Comment [A7]: These were not included with the submission, please double check. Please add all materials used to the table of materials.

All tables (table 1 - 7) have been uploaded together with the revised manuscript. Tables were missing in the original submission due to an uploading error.

Materials used have been added to table of materials.

Comment [A8]: This note is redundant, Please see the line above and make steps crisper.

Redundant parts has been removed. Cloning of targeting vector with homology arms has been divided into substeps (Steps 1.2.2.2 to 1.2.2.3) and steps have been clarified.

Comment [A9]: Reference for how to do this?

Homology arms are cloned into targeting vector by restriction enzyme cloning. This information has been added to step 1.2.2.3 and a reference for restriction enzyme cloning has been added (reference 14 and 15).

Comment [A10]: Some parts are action steps and can be converted to steps and substeps in imperative tense.

Action steps have been added to step 1.2.2.2. and 1.2.2.3.

Comment [A11]: To adhere to JoVE’s poicy oon avoiding commercial biases, please replace this with a generic alternative. We have indicated all such instances in red font.

Commercial language has been removed and replaced with generic terms.

Comment [A12]: Which medium? RPMI?

Transfection reactions are set up with RPMI with reduced serum concentration optimized for transfection (Gibco Opti-MEM, see table of materials). This information has been added.

Comment [A13]: How and when are the cells counted?

Cells are counted after pooling and before centrifugation and washing (line 221 of revised manuscript)

Comment [A14]: Wash how? Mention centrifuge speed and duration.

After FACS sorting, wash cells once by adding 20 mL of RPMI w/ AB to sorted cells and centrifuge for at 300 x g for 4 min at RT. This has been added to step 2.2.3.

Comment [A15]: This was note defined so far.

“RPMI w/ AB” is defined in step 2.2.2.

Comment [A16]: Where is this from ? Did you generate it (provide the step number)?

The reporter sequence is assembled (cloned) in step 1.2.2.1. In our example we used HIV-derived long terminal repeat sequence (LTR), followed by tdTomato fluorescent reporter sequence and BGH polyA-sequence. However, experimenters are free to choose components of reporter and targeting vector depending on research question. Assembly of reporter sequence is described in 1.2.2.1. Components of reporter sequence (LTR, tdTomato sequence, BGH-PolyA) and rationale of reporter design is introduced in introduction (line 90 – 92 of revised manuscript) and in step 1.

Comment [A17]: What do you analyze? Mention gating strategy if relevant.

Cells are analyzed for fluorescent reporter gene expression and gated on viable single cells based on size in forward and sideward scatter. This information has been added to step 2.3.4.

Comment [A18]: Do you plate them after sorting? How? Do you perform FACS sorting again after 10-14 days?

Cells are not sorted a second time. After sorting in step 2.2.3, cells are cultivated for 10 to 14 days (step 2.2.4) and analyzed by FACS and PCR after these 10-14 days of expansion. This has been clarified in step 2.3.1. and in the Note after step 2.3.4.

Comment [A19]: Please provide the step number for greater clarity.

Cells from step 2.2.4 after expansion of 10 – 14 days are used for gDNA preparation. This has been added to step 2.4.1.

Comment [A20]: Need reference or details

Design of primers for integration junction PCRs is described in the Note after Step 2.4. (line 266 – 272 of revised manuscript). Primers have to be designed by the experimenter depending on the chosen integration site and reporter sequence. PCR reactions and cycling conditions are provided in table 3 and 4.

Comment [A21]: Not provided.

Tables 1 – 7 are provided with the revised manuscript. Tables were missing in the original submission due to an uploading error.

Comment [A22]: Which cells?

Cells from step 2.2.4 after expansion are used for generation of single cell clones. This information has been added to step 3.1.2.

Comment [A23]: Mention how and when cells are counted.

Cells from step 2.2.4.are counted 10 – 14 days after expansion and used for single cell plating subsequently.

Comment [A24]: Mention magnification

Magnification (4x) is mentioned in step 3.1.6.

Comment [A25]: Already containing 100 uL RPMI w/AB?

Cells are transferred to a 96- well plate containing 100 µL medium. 100 µl of this cell suspension is transferred to an empty plate, to duplicate the plate. This has been clarified in step 3.1.7.

Comment [A26]: Mention primers and reacton conditions.

Primers and reaction conditions are mentioned in step 3.2.10 and following. The note was intended to explain the time frame of Step 3.2.: Single cell clones have been picked and transferred to new 96-well plate and 96-well plate has been duplicated (step 3.1.6 – 3.1.8). Single cell clones on ‘stock plate’ are now expanding, while in the meantime ‘duplicate plate’ is used to screen single cell clones. This is described in detail in the following steps (3.2.1 – 3.2.12)

To clarify, we have referenced the corresponding parts of the protocol (screening PCR step 4.2.4 – 3.2.12 and flow cytometry step 3.2.2. – 3.2.3) in the note in line 348 – 350 of the revised manuscript.

Comment [A27]: Mention gating /analysis strategy.

Preparation for flow cytometry and gating strategy is explained in step 3.2.3. Please see also answer to comment A26.

Comment [A28]: Mention gating approach.

Viable single cells are gated based on size in forward and sideward scatter and fluorescent reporter gene expression is analyzed. This information has been added to step. 3.2.3.

Comment [A29]: Unclear what is done and what we would film

The reporter sequence contains one fluorescent reporter gene (tdTomato). Expression of fluorescent reporter is analyzed in step 3.2.3. The backbone of the plasmid used for target vector cloning might also contain a second fluorescent reporter gene, for example GFP, based on the chosen plasmid (this is explained in Note following step 1.2.2.3). If fluorescent expression resulting from target vector backbone is detected by FACS in step 3.2.3, this indicates unwanted integration of backbone sequences and corresponding clones have to be excluded from further analysis (step 3.2.13).

We unhighlighted this section for the script.

Comment [A30]: We cannot film designing the primers, but I am wary of unhighlighting this as it will lead to a discontinuity. Please edit the step to add filmable actions if possible.

One could show a screen showing the annotated sequence of the reporter sequence and the position of the primer pairs and length of PCR products *in silico*. We use CLC workbench software for this purpose.

Comment [A31]: Not provided

All tables have been provided with the revised manuscript.

Comment [A32]: Please provide all tables in the revised submission as individual excel files.

All tables have been provided with the revised manuscript.

Comment [A33]: Specifcally which size?

The design of the reporter to be targeted (choice of fluorescent reporter, promoter, additional elements like polyA-sequences) is up to the experimenter, depending on research question. Therefore primer pairs for screening and backbone PCR (step 3.2.10) have to be designed individually as well. A concrete size of PCR products cannot be given in the protocol for screening and backbone PCR. For a positive result, PCR products should show the corresponding specific size. This has been clarified in the note following step 3.2.12.

For positive control PCR, primer sequences are given in table 5. PCR products should show a specific band of 630 bp. This information has been added to ‘note’ following step 3.2.12.

Comment [A34]: Specifcally which size?

See answer to comment A33.

Comment [A35]: Not filmable so i have unhighlighted. We can sometimes include brief non-filmable steps at the conclusion of the protocol section.

One could explain in one sentence in the end as a conclusion, that clones have been screened by PCR and flow cytometry, and those which show positive results in both screens can be selected for further analysis.

Comment [A36]: I split this up, please verify and edit accordingly.

The edit has been verified and confirmed.

Comment [A37]: What is the size again here?

For analysis, PCR product sequences are aligned to expected sequences after Sanger sequencing. This has been clarified in step 3.3.5.

Sizes of PCR products in step 3.3.4 cannot be given, as PCR primers are designed individually by the experimenter based on targeted locus and reporter sequence. An example is given in representative results (Figure 4a).

Comment [A38]: Please replace the commercial names.

Commercial names (HighPrime in step 3.2.13.2 and illustra ProbeQuant in step 3.4.13.3.) have been replaced.

(this part of the protocol has been moved to supplementary protocol. Comment [A38] shows up as Comment [A1] in supplementary protocol)

Comment [A39]: These need to be referenced in-text somewhere. Please upload all tables with your submission.

All tables have been uploaded with the revised manuscript. Table 6 and 7 are referenced in step 3.2.11 (screening PCR).

Recipes for PCR reactions vary depending on the polymerase and supplied buffer systems used (Phusion polymerase, TaqPlus precision polymerase etc.). We have used three different commercial polymerases in this protocol, each of which was tested extensively and showed to work best for the corresponding part of the protocol (amplification from gDNA or plasmid DNA, amplification of long fragments, more robust or more sensitive polymerases). We would like to share our experience which polymerase to use for which application, as this took some time and effort for optimization. We have now deleted commercial language from table headlines (Phusion polymerase, TaqPlus precision polymerase), but have stated the name of the polymerase in the table itself to make this information available to the experimenter.