**From:** "Nam Nguyen" <[em@editorialmanager.com](mailto:em@editorialmanager.com" \t "_blank)>

**Subject: Your JoVE Submission JoVE52610R1**

**Date:** October 6, 2014 at 1:35:22 PM PDT

**To:** "James I Mullins" <[jmullins@u.washington.edu](mailto:jmullins@u.washington.edu" \t "_blank)>

**Reply-To:** "Nam Nguyen" <[nam.nguyen@jove.com](mailto:nam.nguyen@jove.com" \t "_blank)>

CC: [aleksandra.jachtorowicz@jove.com](mailto:aleksandra.jachtorowicz@jove.com" \t "_blank)  
  
Dear Dr. Mullins,  
  
Your manuscript JoVE52610R1 'Pairwise Growth Competition Assay for Determining the Replication Fitness of Human Immunodeficiency Viruses' 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.   
  
Please use the "track-changes" function in Microsoft Word as you revise your manuscript text to address these comments. When you have revised your submission, please upload the revised document along with an additional word document with individual responses to each of the editorial and peer review comments below. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.  
  
**Your revision is due by Oct 20, 2014. Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays.**  
  
To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove" \t "_blank) and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.  
  
Sincerely,  
  
Nam Nguyen, Ph.D.   
Science Editor  
[JoVE](http://www.jove.com/" \t "_blank)  
1 Alewife Center, Suite 200, Cambridge, MA 02140  
tel: [617-674-1888](tel:617-674-1888" \t "_blank)

**Editorial comments:**  
  
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52610\_R1\_090314.docx) is located in your Editorial Manager account. Please download the .docx file and use this updated version for any future revisions.   
  
Changes made by the Science Editor:  
  
1. There have been edits made to the manuscript.   
  
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.  
  
2. Please recheck all in-text protocol references. There were references to step numbers that do not exist as in step 1.3.3.

*Step 1.3.3 refers to existing step 1.2.2 to 1.2.6*  
  
3. Please revise the dilution instructions in step 4.5 and 4.7 for clarity. “Dilute the antibody to \_\_\_\_ with the diluent”

*To clarify, additional steps were added (4.1). The step 4.5 and 4.8 were rewritten.*

4. In step 5.1.6, what is used to wash the cultures? Wash buffer?  
*Wash with fresh cell media. To clarify, the step 5.1.7 to 5.1.9 are now 5.1.6.1 to 5.1.6.3*  
  
**Reviewers' comments:**  
  
**Editor's Note:** Due to the enthusiastic response to the peer-review invitations, your manuscript has seven reviews instead of the typical three. Instead of censoring the reviews, the reviews are all given in its entirety.   
  
**Reviewer #1:**   
*Manuscript Summary:*   
The authors have provided a manuscript detailing procedures for conducting HIV-1 fitness/competition assays which have been previously used to examine fitness cost of immune escape mutations and could be used to study the basic replication effects of almost any mutation in the HIV-1 genome. Many different approaches have been published for conduct of viral in vitro competition experiments, particularly within the field of HIV research. Having a highly detailed description and video of the procedures would provide a benefit to the HIV-1 scientific community by helping to promote the establishment of transparent standards for conduct of HIV fitness assays. The approach is generally sound and thoroughly described but could benefit from some additional discussion and clarification, as described in comments below.  
  
*Major Concerns:*  
1. "PBMC" is used generically in the manuscript as if PBMCs are a cell line. Human PBMCs can differ massively between different individuals in susceptibility to HIV infection and replication in vitro. What source of PBMCs did the authors use: established donors, blood bank, other? What is recommended for labs attempting to adopt this technique? It seems to me that known established donors with demonstrated consistent susceptibility to in vitro infection would be highly beneficial to achieve consistent results.  
*For all experiments, we used PBMCs from a single donor who has demonstrated consistent susceptibility to in vitro infection. This point is now clarified in the discussion as follows:*

*“The protocol for determining the TCID50, viral growth kinetics, and for performing the growth competition assays were optimized using HIV-1 subtype B, NL4-3 COTB-p24, and PBMCs from a single donor whose PBMC have demonstrated consistent susceptibility to HIV-1 infection in vitro. The culture period and sampling time points presented in this protocol are likely to be suitable for studying HIV-1 group M viruses in human PBMCs. Using PBMCs from a single source is highly recommended to obtain consistent results as the viral replication can vary in different donor cells35. Although less desirable, PBMCs pooled from multiple donors can be used as a substitution provided that the same pool is used across all experiments. Another alternative is the use of cell lines. The protocol presented here was used successfully with the T-cell line CEMx174 23,36. However, it is important that the numbers of cells seeded are re-optimized to achieve consistent cell growth. Viral growth kinetics must also be re-established, as it is likely to vary in different cell lines, to determine the appropriate sampling time points in the growth competition steps."*

2. Some discussion of optimal infection ratios is warranted. In previous work, the authors have suggested that a 70:30 mutant:WT ratio is optimal for these experiments (Lanxon-Cookson et al 2013 J Virol Meth) but in other similar work have chosen to use 1:1 ratios (Liu et al 2013 J Virol Meth and this manuscript). This manuscript could benefit from a brief discussion of infection ratios to provide clarity.

*The use of infection ratio is added to the discussion as follows:*

*“This protocol suggests an initial infection ratio of 50:50, assuming that the fitness differences of the viruses are generally unknown beforehand. However, the use of unequal input ratios is appropriate when there is preliminary data suggesting significant differences in viral replication kinetics. In these cases, an infection ratio of 70:30 is recommended to allow for the detection of a large fitness difference where the less fit virus is placed in excess9.”*

3. For the virus titration, what is the basis for selecting day 7 as the timepoint to assay presence or absence of virus? The problem with a relatively early timepoint such as this is that a low fitness may take longer than 7 days to replicate to levels detectable by capsid ELISA. In this scenario the low fitness virus will be assigned an artificially low TCID50, which would ultimately result in the virus being assigned a fitness value that is higher, or closer to wild-type, than what is actually true. Please address this issue and justify use of day 7 supernatant for titration.

*Day 7 was chosen based on the longitudinal sampling of the virus we studied. We apologize for not making this point clearer in the protocol. As other HIV strains may exhibit slower viral growth, the protocol is now modified to include longitudinal samplings until the observed infectious titer is stable, as was done in our initial studies.*  
4. One potential pitfall of the described method is that production of HIV virions through transfection results in a high level of defective virions and virions containing altered ratios of viral proteins compared to a natural infection (not driven by an artificial promoter). Some investigators have proposed that viruses produced through transfection should be briefly passaged in a cell line or PBMC to eliminate this problem. This could have particularly strong effects on fitness comparisons of HIV proteins such as env gp120 that may be altered by presence of defective virions or altered levels of protein per virion. It would be prudent to compare fitness of viruses produced through transfection with those briefly passaged through a natural infection cycle. This point is beyond the scope of this particular methods article, but should be addressed in the future.

*We appreciate the reviewer’s excellent point. We have indeed performed passage experiments without noting differences in fitness but agree that additional, future study will be informative regarding the utility of the additional step suggested.*

*Minor Concerns:*  
1. Lines 180 and 218: 10 mM dNTPs is your stock concentration, not final concentration. Please change to final concentration as with other reagents. *Corrected*.  
2. Line 186: "primers primer" - repeat of word *corrected*  
3. Line 295: propagation is misspelled "ropagation" *corrected*  
4. Line 339: Do you really do extended incubations for titration in a round-bottom plate? Don't all the cells form a tight clump in the center limiting cell survival? *Yes, we observed a clump of cells in the center but did not observe defects in cell growth. In addition, the round-bottom allows for greater cell-cell interactions, which encourages viral spread.*   
5. Line 458-459: Please note where this cDNA synthesis primer binds - what is it specific for (presumably HIV NL4-3 Vif)? *HXB2 nucleotide position added*  
6. Line 467: Correct the final reaction concentration for DTT. It cannot be 120 mM since the stock DTT that comes with SuperScriptIII is 100 mM. *corrected*  
7. Lines 488 and 552: Which PCR "master mix" is referred to here? Be specific. *For qPCR master mix, the trademark name is omitted according to the journal policy, however the detail is given in the table of materials. For line 552 (now line 725), the word “master mix” is changed to inoculum to prevent confusion.*  
8. Line 573: primer sequences are not provided *corrected*  
9. Line 658: "is" should be "are" *corrected*  
  
  
**Reviewer #2:**   
*Manuscript Summary:*   
Manocheewa et. al. describe a method for the generation of tagged mutant HIV viruses and their subsequent characterization through a pairwise competition assay. The paper is well written, with only a few minor queries or concerns.  
  
*Major Concerns:*  
No major concerns.  
  
*Minor Concerns:*  
1.2.1 line 220/221 - 35 cycles is a large number for a quickchange reaction. It is more usual to have 18 cycles to help avoid introducing mutations. Was this step optimized? (also relevant to section 1.3.2) *The number of PCR cycles stated in step 1.3.2 was a typo and is now corrected to 25 instead of 35. In addition, yes, this step was optimized using Phusion high fidelity DNA polymerase. We obtained better yields with a higher number of cycles. We also did not detect extra mutations after sequencing the full-length of HIV-1 coding region.*

1.2.5 As quickchange mutagenesis has been used to introduce the mutations, ideally the whole HIV-coding region should be sequenced to ensure no additional mutations have been introduced, particularly due to the large number of cycles used (re 1.2.1). *We agree that it would be ideal to sequence the whole HIV coding region and we had done so during the protocol development. We did not detect any extra mutations and, hence, modified the protocol to increase efficiency. This point is added to the discussion.*

*“For PCR-based cloning and site directed mutagenesis, the use of a high-fidelity DNA polymerase is crucial to reduce the likelihood of extra mutations. Use of the minimal number of PCR cycles needed to yield adequate amounts of products is also recommended. In our experience, we did not detect any extra mutations after the PCR-based cloning and site-directed mutagenesis steps described here. Nevertheless the PCR products should be sequenced to check for any undesired mutations. Ideally, the entire HIV coding region should be resequenced.”*

4.1.4 The authors have described an ELISA assay, however the Reed & Muench method is for calculating Tissue-culture infectious dose (TCID50). It is not completely clear how the ELISA results translate to the Reed & Muench calculation - is the ELISA output Binary (a well is infected or not-infected) - which would make sense for the TCID50 calculation, or analogue - a well can be more or less infected? This section just needs rewording in order to clarify this - some guidance for the reader on typical infected or mock A450-600 values could also be beneficial. *We apologize for the confusion. The ELISA output was interpreted as a binary (infected or uninfected) based on the amount of p24 compared to the background signal detected in the negative control or mock wells. We have edited the protocol to clarify this.*  
  
Discussion:   
The method used can suffer from some sensitivity issues, however the material produced for the qRT-PCR or peak height comparison is also usable for other approaches such as bulk sequencing of individual clones. Other approaches could be discussed briefly in the discussion section. *This point was added to the discussion as follows:*

*“The PCR products produced for RT-qPCR and Sanger sequencing can also be use with other methods to determine viral ratio such as bulk sequencing of individual clones 12,13, HTA 4,7,17,19, or oligonucleotide ligation assay (OLA)38.“*  
  
Filming comments:  
I understand the authors need to be selective in the aspects of the paper chosen to film for the paper, but some current omissions would be beneficial if they could be included. Some of the features not currently being filmed that could benefit include 5.2.3, 5.3.1, and 6.11.4. (a number of the currently highlighted regions would likely be represented in the final video by slides, rather than filming per se so it may be possible to fit this within the limit).

*We appreciate the reviewer’s concern and suggestion on this matter. We have modified the selection of steps to be included in the filming.*   
  
Figures:  
Fig 1. In the fitness assay section, 'Real time PCR' and 'Sequence chromatogram' aren't aligned. *Fixed*  
  
Fig 4A. It would be beneficial to include the control virus sample on the graph.   
*Added*  
  
**Reviewer #3:**   
*Manuscript Summary:*  
This manuscript describes a methodology to assess the in vitro replication fitness of mutant HIV-1 strains during mono- and dual-infections. The manuscript is clearly written and easy to follow.   
  
*Minor Concerns:*  
1) Regarding sections 5.2 and 5.3: "RT PCR" abbreviation can be used for both Real Time PCR and Reverse Transcription PCR. To avoid confusion, the Reverse Transcription- quantitative PCR described here can be abbreviated as 'RT-qPCR'.  
*We have modified the acronym of our method as suggested.*

2) Section 2.3: The formula can be revised as   
' Total volume of DMEM in µl = 100 µL - DNA volume in µl.' *We have modified the text as suggested.*  
  
3) Section 6.11.1: The given primer sequence (5'-NNN-3') implicates use of Random trimers. If it is not the case, the sequence information can be removed. *We apologize for the mistake. The primer sequences are provided.*  
  
4) Discussion: Regarding PCR based cloning, a discussion on introduction of random mutations by DNA polymerase enzymes (error rate and GC-rich templates) and steps to minimize this undesired event (high fidelity/proof reading capability), can be included.

*This point was addressed above in response to Reviewer #2 (Minor concern, section 1.2.5)*  
  
**Reviewer #4:**   
*Manuscript Summary:*   
The manuscript by Manocheewa et al. thoroughly describes the methodology to conduct pairwise competition assays for HIV. All the steps relevant for the process are rigorously explained, from the initial cloning and rescue of HIV variants to the final analysis of the data from the growth curves. The authors also provide interesting comments on the possible use of alternative techniques or reagents when necessary, a most welcome supplementary piece of information that allows the protocol to be used by a variety of laboratories with varying resources. The methodology described can also be easily adapted to the study of many other viruses, not only HIV. Overall, the manuscript is perfectly suited for this kind of journal in its present state.  
  
*Major Concerns:*  
None  
  
*Minor Concerns:*  
- Several abbreviations are not defined in the text (e.g. PBMC, PHA) these have been *corrected*  
  
- Figure 4 would benefit from a scheme to help explaining the two different analysis methods proposed *An additional figure showing the workflow from the growth competition samples to the result, viral ratios, is added as Figure 4B. The previous figure 4B is now 4C*  
  
  
**Reviewer #5:**   
*Manuscript Summary:*   
The submitted manuscript provides a succinct and complete protocol for the creation of mutant HIV infectious clones and a guide for performing competition assays to compare the effect of such mutants. Construction of chimeric HIV-1 molecular clone mutants details a process that could be considered basic molecular cloning knowledge, however for the sake of completeness it is a good primer. After virus generation and quantification by p24 ELISA, the author nicely details the steps necessary for measuring the exponential growth rate of both individual and competitive viral infections. Several portions, especially involving the mathematical calculations necessary for chromatogram comparison and growth rate estimation are distinctly lacking (detailed below), however given that these can be easily defined and elaborated, I recommend this manuscript for publication in the Journal of Visualized Experiments.  
  
*Major Concerns:*  
\* Step 6.11.4-6.12: More details are required for how to access and utilize the ChromatQuant and GRC web tools. *More details have been added, including the URL of the web tools.*

\* Line 612 and Figure 3c: Viral growth rate is labeled on figure 3c as "g" units with no reference as to how this dimensionless unit is calculated. *Additional description and reference are now given in step 5.4.2.*  
*Minor Concerns:*  
\* Line155: Do the aa point-mutants have some president or an expected effect? *T242N was previously reported to have a moderate fitness cost, similar to the result shown in this study. The fitness cost of the other two mutations had not been reported previously, as now noted in the text.*

\* Step 1.2.3.1: specify culture volume *added*

\* Steps 2.2 and 2.3 should be part of step 2.5; calculating transfection mixture volumes. *Corrected*

\* Step 2.4: Please include which steps you are referring to in the note? Is this dependent on how much plasmid you have, or just how many infectious clones you want to grow? *The number of seeded wells depends of the volume of viral stocks desired, which in turn depends on the number of experiments planned. However, the user must ensure that the adequate amount of plasmid DNA is prepared before starting the transfection. The descriptions of step 2.1 to 2.3 are modified to better clarify this point.*

\* Step 3.3: "Seed 2x104 PHA stimulated PBMC/well in 100 µl/well cIMDM in a round bottom 96-well plate." This math seems incorrect. 100 uL of a 2e6 cell/mL solution will deliver 2e5 cells. *Corrected*

\* Step 3.4: "Make a dilution series of the viral stock in a 96-well master plate using 12, 3-fold serial dilutions. Starting from a 10-fold initial dilution…" Further explanation might help here. For example: "By adding 15 uL of virus stock to 135 uL media in the first well, and then transferring 50 uL into 100 uL for subsequent wells…" *The description of this step is modified and an explanation is added as follows:*

*“3.4) Make a 1:10 dilution of the viral stock. Then, from the first diluted stock, make twelve 3-fold serial dilutions in a 96-well master plate. This dilution scheme is recommended for detecting viral titers in the range of 104 to 106 infectious unit (IU) per ml. For example, add 20 µl of virus stock to 180 µl media in the 1.5 ml tube. Mix the dilution by pipetting carefully. Then transfer 90 µl of the diluted stock into 180 µl media of the first well and mix well by pipetting. Continue dilution series by transferring 90 µl from the current well to 180 µl media in the next well eleven more times. Increase or decrease the initial dilution if titers higher than 106 IU/ml or lower than 104 IU/ml are expected, respectively.”*

\* Step 3.5: should the inoculum be added to the media already in the well or replaced. *Inoculum should be added to the seeded PBMCs. The sentences are revised to clarify this as follows:*

*“3.5*) Add 40 µl of the serially diluted viral stock from the master dilution plate to the seeded PBMCs plate (from step 3.3) in quadruplicate. Incubate plates at 37 oC in a 5% CO2atmospherefor 16-24 hr.”

\* Step 6.4: Can a comparison be made between monoinfection kinetics and competition growth rates? This might be complicated when using half of each virus. *We did not compare the growth rate between monoinfection and growth competition, because, as the reviewer suggests, the conditions, MOI and presence or absence of the other virus, are different.*\* Figure 3B: You should explain why the measured cDNA copy number decreases between day 0 and 2. *The explanation is added as follows:*

*“The decrease in cDNA copy number between day 0 (corresponding to the inoculum) and day 2 is due to the absorption of virus to cells and the removal of unbound virions by the day 1 wash (step 5.1.6).”*  
  
**Reviewer #6:**   
*Manuscript Summary:*   
This submission includes many techniques required to create a pairwise comparison of fitness of HIV variants. The background is well researched.   
  
*Major Concerns:*  
The biggest concern with this submission is the parts that are chosen to be videotaped. The value of the JoVE article for the user is the demonstration of the technique. Many of the yellowed lines (which I assume are for taping) do skip important steps. While it is important that only 30 steps be included in the video, the authors should consider very carefully which steps they cut out. Does the procedure make sense without them? I am not sure that I could do the procedure based on the chosen 30 steps. It is possible to combine some steps so that they can be taped and will make for a better flow. *We very much appreciate the concern and have modified our selection of steps to be videotaped.*   
  
*Minor Concerns:*  
1.1.1 and 1.1.1.1 the procedure refers back to these steps several times. It is unclear in 1.1.1 what "design" means. What is the experimental process? *By design we mean defining the primer sequence. More explanation is added.*   
  
line 295 - should that be propagation??? not ropagation? *Typo corrected*  
In many steps the authors refer to "manufacturers protocol". It would be helpful to have manufacturer listed at that step so the reader does not have to search through the extensive reagent list. *The exclusion of manufacturer names was done according to journal requirements.*  
  
1.2.4 mentions double restriction enzymes - which enzymes?? *The appropriate restriction enzymes will change according to the insert fragment DNA sequence. Hence, specific enzymes cannot be given in advance.*   
  
1.2.2 - which methylation competent bacterial strain did you use? *We used the commercially Top10 Escherichia coli. This information is added*  
  
3.4 The sentence is awkward (starting at 343) *We modified the sentence and now provide an example.*  
3.8 - It is unclear how you determine infectious titer from an ELISA for the p24 antigen. *Further explanation is given in step 4.*  
  
4.14 - How do you calculate the titer using Reed-Meunch? *More description is added as follows:*

*“Read absorbance at 450-650 nm in each well using a microplate reader. Use the absorbance value to score each well as infected or uninfected. A well is considered to contain infectious virus if the absorbance value is at least three times higher than the value read from mock / negative control wells. Calculate TCID50 of the viral stock using the Reed-Meunch method31.”*  
  
**Reviewer #7:**   
*Manuscript Summary:*   
The paper from Manocheewa SI, et al clearly describes the process to generate a new set of recombinant/mutant viruses, test for their growth capabilities and finally calculate the fitness by two methods (sanger sequencing peak determination or qRTPCR). The methods are well explained with detailed information. I appreciate the inclusion of a flow diagram (Figure 1) to easily follow each step. The representative results they provide is clear enough to understand what can be expected as result.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
On line 295, it should say "propagation" instead of "ropagation" *Typo corrected.*  
  
On point 6.4 (line 524), I would appreciate if they authors added that the final ratio is 1:1 between both viruses, and if they could discuss the situations in which you would use a different ratio. i.e: when one mutant/variant displaces the other too fast to calculate a fitness. *This point is added to the discussion.*

*“This protocol suggests an initial infection ratio of 50:50, assuming that the fitness differences of the viruses are generally unknown beforehand. However, the use of unequal input ratios is appropriate when there is preliminary data suggesting significant differences in viral replication kinetics. In these cases, an infection ratio of 70:30 is recommended to allow for the detection of a large fitness difference where the less fit virus is placed in excess9.”*

Figures 3B and 3C are badly scaled compared to the rest of the figure (too small). *We adjusted all graph figures to be the same size and used the same font size*  
  
Page 28 and onwards have weird text showing at the bottom. Care should be taken with this.