Journal of Visualized Experiments

Pairwise Growth Competition Assay for Determining the Replication Fitness of Human Immunodeficiency Viruses

--Manuscript Draft--

Manuscript Number:	JoVE52610R2
Full Title:	Pairwise Growth Competition Assay for Determining the Replication Fitness of Human Immunodeficiency Viruses
Article Type:	Methods Article - JoVE Produced Video
Keywords:	HIV-1; Recombinant; Mutagenesis; Viral replication fitness; Growth competition; Fitness calculation
Manuscript Classifications:	2.4: Viruses; 3.2: Virus Diseases; 5.5.240.617: Tissue Culture Techniques
Corresponding Author:	James I Mullins UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	jmullins@u.washington.edu
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Siriphan I Manocheewa
First Author Secondary Information:	
Other Authors:	Siriphan I Manocheewa
	Erinn C Lanxon-Cookson
	Yi I Liu
	J. Victor I Swain
	Ushnal I Rao
	Brandon I Maust
	Justine I Sunshine
	Moon I Kim
	Morgane Rolland
	Wenjie Deng
Order of Authors Secondary Information:	
Abstract:	In vitro fitness assays are essential tools for determining viral replication fitness for viruses such as HIV-1. Various measurements have been used to extrapolate viral replication fitness, ranging from the number of viral particles per infectious unit, growth rate in cell culture, and relative fitness derived from multiple-cycle growth competition assays. Growth competition assays provide a particularly sensitive measurement of fitness since the viruses are competing for cellular targets under identical growth conditions. There are several experimental factors to consider when conducting growth competition assays, including the multiplicity of infection (MOI), sampling times, and viral detection and fitness calculation methods. Each factor can affect the end result

	and hence must be considered carefully during the experimental design. The protocol presented here includes steps from constructing a new recombinant HIV-1 clone to performing growth competition assays and analyzing the experimental results. This protocol utilizes experimental parameter values previously shown to yield consistent and robust results. Alternatives are discussed, as some parameters need to be adjusted according to the cell type and viruses being studied. The protocol contains two alternative viral detection methods to provide flexibility as the availability of instruments, reagents and expertise varies between laboratories.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

TITLE:
Pairwise Growth Competition Assay for Determining the Replication Fitness of Human Immunodeficiency Viruses

AUTHORS:
Manocheewa, Siriphan
Department of Microbiology
University of Washington
Seattle, WA
manocs@uw.edu

Lanxon-Cookson, Erinn C.
Department of Microbiology
University of Washington
Seattle, WA
erinn_lanxon@yahoo.com

Liu, Yi
Department of Microbiology
University of Washington
Seattle, WA
Yiliu197@uw.edu

Swain, J. Victor
Department of Microbiology
University of Washington
Seattle, WA
jvicswain@gmail.com

Rao, Ushnal
Department of Microbiology
University of Washington
Seattle, WA
ushnal@yahoo.com

Maust, Brandon
Department of Microbiology
University of Washington
Seattle, WA
bmaust@uw.edu

Sunshine, Justine E.
Department of Microbiology
University of Washington
Seattle, WA
justinesunshine1@gmail.com

Kim, Moon
Department of Microbiology
University of Washington
Seattle, WA
Moonk87@uw.edu

Rolland, Morgane
U.S Military HIV Research Program
Walter Reed Army Institute of Research
Silver Springs, MD
mrolland@hivresearch.org

Mullins, James I.
Departments of Microbiology, Medicine and Laboratory Medicine
University of Washington
Seattle, WA
jmullins@uw.edu

CORRESPONDING AUTHOR:
Mullins, James I., Ph.D.
Phone: 1-206-732-6163
Fax: 1-206-732-6167

KEYWORDS:
HIV-1; Recombinant; Mutagenesis; Viral replication fitness; Growth competition; Fitness calculation

SHORT ABSTRACT:
Growth competition between nearly isogenic viruses provides a sensitive measurement for determining relative replication fitness. The protocols described here include the construction of recombinant HIV-1 clones, virus propagation and growth competition and analysis methods optimized to yield sensitive and consistent results.

LONG ABSTRACT:
In vitro fitness assays are essential tools for determining viral replication fitness for viruses such as HIV-1. Various measurements have been used to extrapolate viral replication fitness, ranging from the number of viral particles per infectious unit, growth rate in cell culture, and relative fitness derived from multiple-cycle growth competition assays. Growth competition assays provide a particularly sensitive measurement of fitness since the viruses are competing for cellular targets under identical growth conditions. There are several experimental factors to consider when conducting growth competition assays, including the multiplicity of infection (MOI), sampling times, and viral detection and fitness calculation methods. Each factor can affect the end result and hence must be considered carefully during the experimental design. The protocol presented here includes steps from constructing a new recombinant HIV-1 clone to

performing growth competition assays and analyzing the experimental results. This protocol utilizes experimental parameter values previously shown to yield consistent and robust results. Alternatives are discussed, as some parameters need to be adjusted according to the cell type and viruses being studied. The protocol contains two alternative viral detection methods to provide flexibility as the availability of instruments, reagents and expertise varies between laboratories.

INTRODUCTION:

Viral replication fitness is defined as the capacity of a virus to produce infectious progeny in a given environment[1] and is an important contributing factor in determining the prevalence of a virus variant at the population level over time[2]. As *in vivo* fitness studies are not feasible with pathogenic human viruses, such as HIV-1, various *in vitro* and ex *vivo* replication fitness assays have been developed to study the effects on fitness arising from drug resistance and immune escape mutations, epistasis and the evolution of viral populations[3-6]. Among different fitness assays, growth competition assays are recognized to yield more sensitive and valid measures of fitness differences, as two or more viral variants compete for the same cell population under precisely the same environmental conditions, as occurs *in vivo*[1,7,8]. Before starting growth competition experiments, several variables need to be determined, including the use of different multiplicities of infection (MOI), viral input ratio, and timing of sampling for analysis. We have studied the effects of these parameters on viral growth kinetics and on the outcome of competition experiments, and have identified key factors necessary for robust measurements of HIV-1 fitness in cell culture[9].

In addition to assay variables, there are a variety of methods for quantitating viral variants in growth competition experiments. Bulk[10,11] or clonal sequencing[12,13] has been used to determine the ratio of the competing viruses based on the nucleotide frequencies at the site(s) of interest. Relative fitness is derived from changes in this ratio over time. This method is convenient as DNA sequencing services are widely available. The parallel allele-specific sequencing (PASS) method enables sequencing at multiple sites and the detection of recombinants[14], but it also requires specifically developed reagents and detection systems. Essentially, these methods were developed to study viral strains with a small number of nucleotide differences in a region of interest. Other methods use a small reporter gene[15] or synonymous mutations[4,16-18] as tags to distinguish the competing viruses by sequencing, heteroduplex tracking assays (HTA)[4,7,17,19] or reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)[15,16,18,20], all of which can be made applicable to study competing strains regardless of sequence similarity. An additional step is required to introduce tags into viral genomes and the RT-qPCR assay also requires specific reagents and instrumentation. We have found that bulk Sanger sequencing yields comparable results[9].

Following growth competition, viral replication fitness is presented as relative fitness, or a fitness ratio between two viral variants. The relative fitness of a virus can be defined as the final proportion of a viral variant normalized by its initial proportion in the inoculum or as the net growth rate difference between the two competing viruses. We

found that the latter method, using longitudinal data points only within the exponential growth phase, produced the most robust results[9,20].

In vitro fitness assays are used primarily to study biological clones[6-8] and infectious molecular clones of HIV-1. The latter, being amenable to genetic manipulation, are often employed to study the effect on fitness from particular mutations or specific sequences of interest[3-5,21,22]. The following protocols describe a workflow from the point of constructing new full-length infectious HIV-1 molecular clones using HIV-1 vectors containing a sequence tag, introducing mutations of interest, making viral stocks and establishing viral growth kinetics, to performing the growth competition assay and calculating relative fitness (**Figure 1**).

Using our optimized procedures, we created three recombinant HIV-1 mutants and determined their replication fitness. The recombinant molecular clone was first constructed by replacing the HIV-1 *gag-p24* gene region of pNL4-3, a plasmid containing a full length infectious genome of HIV-1 lab strain NL4-3, with a synthetic COTB (Center-Of-Tree, subtype B) *gag-p24* sequence[23] to create the prototype strain. Single amino changes (T186M, T242N, and I256V) were then introduced to create three mutant clones. Each mutant was competed against the prototype virus to observe the fitness impact of each mutation in the given genetic background. The three mutants demonstrated varying levels of replication fsitness from slight to significantly lower than the prototype virus. The T242N mutation was previously reported to have a moderate fitness cost [24-26], similar to the result shown in this study. The fitness cost of the other two mutations had not been reported previously.

PROTOCOL:

The protocol, as described below, does not include any patient identifiable information and is thus not considered Human Subjects Research by the University of Washington Institutional Review Board or Human Subjects Division.

1) Construction of chimeric HIV-1 NL4-3 molecular clones

1.1) Amplify insert DNA fragment

1.1.1) Design chimeric primers. The 5’ halves of both forward and reverse primers contain an HIV-1 vector sequence, at which the fragment will be inserted. The 3’ half of the primers must contain the end of the insert sequence (**Figure 2**). Make sure that the chimeric primer sequence retains the original open reading frames.

1.1.1.1) Use primers at least 20 bases in length, with a melting temperature greater than or equal to 60 °C, ~50% GC content, and a low tendency to form primer dimers, heterodimers and/or hairpin structures. Assess these properties using the OligoAnalyzer web tool (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

1.1.2) Use PCR[27] and the chimeric primers to amplify insert DNA (**Figure 2**). For each PCR reaction, use 1X high fidelity buffer, 0.2 mM dNTPs, 1 U of high fidelity DNA polymerase, 0.5 µM of forward chimeric primer, 0.5 µM of reverse chimeric primer, and 1 pg - 10 ng of DNA sample carrying insert region. Add dH_2O to a final volume of 50 µl.

1.1.3) Set thermal cycling steps as follows: Perform an initial DNA denaturation step at 98 °C for 10 sec. Amplify with 30 cycles of DNA denaturation at 98 °C for 10 sec and DNA annealing at 3 °C above the lowest melting temperature of the two primers for 20 sec. Perform a final extension at 72 °C for 10 min. Store PCR products at 4 °C.

1.1.4) Take 5 µl of the PCR products from the previous step and run agarose gel electrophoresis[28].

1.1.4.1) Use a 0.7% agarose gel, 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA), 0.5 µg/ml ethidium bromide (EtBr) final concentration and 1 kb ladder as the DNA size marker. Set power source voltage to 5 V/cm distance between electrodes. Stop the electrophoresis when the loading dye migrates through about 2/3 of the gel length. Visualize the gel using a gel documentation system[28].

Note: EtBr is a suspected carcinogen and must be properly disposed of, per institution regulations. Gloves should always be worn when handling gels containing EtBr. Change to new gloves after finish handling EtBr containing material and before handling other materials or equipment to prevent cross contamination.

1.1.5) If only one DNA band with size corresponding to the desired PCR product is detected, purify the rest of the PCR product using a commercial kit such as QIAquick PCR Purification Kit according to the manufacturer's protocols.

1.1.5.1) If other, non-specific bands are also present, use the rest of the PCR product to run preparative gel electrophoresis. Use the same parameters and conditions specified in step 1.1.4. Ensure that the gel well is large enough to load ~45 µl of PCR products. Cut out the band of interest and extract the DNA from the gel using QIAquick gel extraction kit according to the manufacturer's protocols.

1.2) Introduce the insert fragment into full-length infectious HIV-1 subtype B vector (pNL4-3)

1.2.1) Use purified PCR products from step 1.1.5 as PCR primers. Use pNL4-3VifA[20] as template DNA in one PCR reaction and use pNL4-3VifB[20] as template in the other reaction (**Figure 2**). For each PCR reaction, use 1X high fidelity buffer, 0.2 mM dNTPs, 2 U of high fidelity DNA polymerase, 500 ng of primer DNA and 50 ng of template DNA in a final volume of 50 µl. Set the thermal cycling parameters to: 98 °C for 30 sec, 35 cycles at 98 °C for 10 sec, 48 °C for 1 min and 72 °C for 10 min, followed by 72 °C for 10 min.

1.2.2) Add 10 U of *Dpn*I to 50 µl of the PCR reaction and incubate at 37 ^{o}C for 1 hr to digest the template DNA. Ensure that the plasmid DNA is isolated from a methylation competent bacterial strain, e.g., TOP10 chemically competent *Escherichia coli*.

1.2.3) Use *Dpn*I digested product to transform competent bacterial cells. Use heat-shock transformation with TOP10 chemically competent *E. coli*, according to the manufacturer's protocol. To select for bacterial cells containing the recombinant plasmid, use Luria Broth (LB) culture plates containing 100 mg/L carbenicillin.

1.2.3.1) Pick ~10 well-separated colonies and grow each separately in 3 ml LB liquid medium containing 100 mg/L carbenicillin and incubate at 30 °C in a shaker overnight.

1.2.3.2) Use QIAprep Spin Miniprep kit to isolate plasmid DNA from the bacterial liquid culture, according to the manufacturer's protocol.

1.2.4) Use double restriction digestion[29] to determine whether the plasmid DNA contains the proper insert. Ensure that one of the restriction sites exists only within the insert region and the other restriction site exists only once in the HIV-1 vector, outside of the insert region.

1.2.4.1) Digest at least 300 ng of plasmid DNA in a 10 µl final reaction volume. Select restriction buffers, incubation temperature and incubation time according to the manufacturer's protocol of the selected restriction enzymes. Take 9 µl of the digested DNA and run gel electrophoresis as described in step 1.1.4. Select recombinant plasmids that have DNA bands of the predicted sizes.

1.2.5) Confirm sequence integrity of the recombinant plasmids by Sanger sequencing. While rare, unwanted mutation(s) can be introduced during the PCR reactions.

1.2.5.1) Sequence both strands of the plasmid DNA. Follow instructions in step 1.1.1.1 to design sequencing primers. In addition, ensure that the forward and reverse sequencing primers anneal at least 50 bp upstream and downstream of the insert region in the recombinant plasmid, respectively.

1.2.5.2) Submit plasmid DNA and sequencing primers to a commercial DNA sequencing service provider. Prepare DNA sample and primers as specified by the service provider.

1.2.6) Make an endotoxin-free stock of the mutated plasmid DNA using an Endotoxin free plasmid DNA kit according to manufacturer's protocol. Prepare at least 1 µg of endotoxin-free plasmid DNA for transfection in the following step.

1.3) Introduce small-scale mutations via site-directed mutagenesis

1.3.1) Design mutagenic primers with overlapping forward and reverse primers containing the desired mutation(s). Position the base(s) to be substituted, inserted, or deleted in the middle of the primers, flanked by 10-15 homologous bases. Follow instructions in step 1.1.1.1.

1.3.2) Use PCR to synthesize mutant plasmids. For each PCR reaction, use 1X high fidelity buffer, 10 mM dNTPs, 2 U of high fidelity DNA polymerase, 0.5 µM of forward mutagenic primer, 0.5 µM of reverse mutagenic primer and 50 ng of chimeric pNL4-3VifB, from step 1.2.6, in a final volume of 50 µl. Set the thermal cycling parameters to: 98 °C for 30 sec, 25 cycles at 98 °C for 10 sec, 48 °C for 1 min and 72 °C for 10 min, followed by 72 °C for 10 min.

1.3.3) Repeat step 1.2.2 to 1.2.6.

2) Generation of viral stock using transfection

2.1) Calculate the amount of viral stock desired and plasmid DNA required. With a viral titer of 10^4 IU/ml or higher, 1.8 ml of viral stock is sufficient for two sets of growth competition assays, including monoinfections, each done in triplicate. For a transfection done in a 6-well plate, about 1.8 ml supernatant is harvested per well. One µg of plasmid DNA is needed for each transfection done in a 6-well plate.

2.2) For each well of a 6-well plate, prepare 100 µl of transfection mixture, e.g., consisting of 1 µl X-tremeGENE 9 transfection reagent (or comparable product), 1 µg of plasmid DNA and serum-free DMEM.

2.2.1) Determine the volume of plasmid DNA needed, using 1 µg plasmid DNA per well. Ensure that the final concentration of the plasmid DNA is at least 50 ng/µl.

2.2.2) Determine how much serum-free medium (DMEM) is needed per well using the formula: Total volume of DMEM in µl = 100 µl - DNA volume in µl.

2.3) Add 10^6 HEK 293T-17 (ATCC) cells/well in 2 ml of propagation medium (DMEM + 10% fetal bovine serum (FBS)) into a 6-well plate. Incubate for 1 hr at 37 ^oC in a 5% CO_2 atmosphere. Seed as many wells as needed (determined in step 2.1).

2.4) To prepare the transfection mixture, aliquot the appropriate volume of serum-free DMEM, as calculated above, into a 1.8 ml polypropylene microcentrifuge tube, and then add the transfection reagent.

2.4.1) Pipette reagent directly into the media solution, do not add it to the plastic surface of the microcentrifuge tube. Add plasmid DNA last. Pipet up and down gently to mix the solution. Incubate for 15 min at room temperature (15 ^oC to 25 ^oC) to allow the formation of transfection complexes.

2.4.2) Add the mixture in a drop-wise manner to cells seeded in the 6-well plate. Gently shake or swirl the wells to ensure even distribution of transfection complexes.

2.4.3) Seal plates with plastic wrap.

2.5) Incubate cultures at 37 ^{o}C in a 5% CO_2 atmosphere for 48 hr.

2.6) Use a pipette to carefully collect and transfer supernatant to a 15 ml tube through a 0.22 µm filter top.

2.7) Use pipette to transfer 250 µl or more of the filtered supernatant to 1.8 ml microfuge tubes with rubber gaskets in the lids.

2.8) Store filtered supernatants at -80 ^{o}C until use.

3) Determine infectious titer of viral stocks on peripheral blood mononuclear cells (PBMCs)

3.1) Stimulate PBMCs with phytohemagglutinin (PHA). Per one viral stock, seed 2 x 10^6 PBMCs in complete Iscove's Modified Dulbecco's Medium (cIMDM; IMDM supplemented with 20 U/ml of human interleukin 2 (hIL-2), 10% fetal bovine serum and 1% penicillin/streptomycin) supplemented with PHA (1.5 µg/ml). Seed PBMCs at 2 x 10^6 cells/ml. Incubate PBMCs at 37 ^{o}C in a 5% CO_2 atmosphere for 72 hr.

3.2) Harvest PHA stimulated PBMCs. Transfer non-adherent PHA-PBMCs to a 50 ml conical tube. Spin tube at 228 x g for 10 min. Carefully remove supernatant without disrupting the cell pellet. Re-suspend the cell pellet to a final concentration of 2 x 10^5 PBMCs/ml in cIMDM.

3.3) Seed 2x10^4 PHA stimulated PBMCs/well in 100 µl/well cIMDM in a round bottom 96-well plate.

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 10^4 to 10^6 infectious unit (IU) per ml.

3.4.1) 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.

3.4.2) 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 10^6 IU/ml or lower than 10^4 IU/ml are expected, respectively.

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 ^{o}C in a 5% CO_2 atmosphere for 16-24 hr.

3.6) Carefully remove 100 µl of supernatant from each well, and replace with 160 µl of fresh cIMDM to a total volume of 200 µl/well. Incubate plates at 37 ^{o}C with 5% CO_2 atmosphere (day 1).

3.7) On days 4, 7, 10 and 13 transfer 100 µl of supernatant from each well to 100 µl disruption buffer (2% TritonX-100 in PBS), and replace with 100 µl of fresh cIMDM. Store the supernatants at -20 ^{o}C.

3.7.1) Keep sampling and adding fresh cIMDM every three days until the titer stabilizes.

3.8) Determine the 50% tissue culture infectious dose ($TCID_{50}$) of the viral stock by p24 ELISA using the day 7 and 13 samples as described in step 4.

3.8.1) If the $TCID_{50}$ obtained from day 13 is clearly higher than the titer from day 7, the virus stock may need a longer time to expand. Repeat the p24 ELISA using later samples until the infectious titers from two sampling time points become stable (or decrease). Select stocks from samples with the highest titers.

4) **ELISA (Enzyme-linked Immunoabsorbant assay) detection of HIV-1 p24 for determining viral infectious titer**

Note: The following protocol was developed using p24 antigen capture plates prepared in our laboratory[30]. Commercial HIV-1 p24 ELISA plate/kits can also be used, following the manufacturer's protocol.

4.1) Prior to working with samples, prepare working stocks of primary antibody (rabbit anti-HIV-1 SF2 p24 antiserum).

4.1.1) Thaw p24 antiserum at room temperature (RT).

4.1.2) Mix 2.5 ml of glycerol with 2 ml of 10% FBS in phosphate buffer saline (PBS).

4.1.3) Add 0.5 ml of antiserum and mix.

4.1.4) Store 1 ml of aliquots at -20 ^{o}C.

4.2) Thaw samples from step 3.7 in a 37 ^{o}C incubator.

4.3) Wash the p24 capture plate 5 times with wash buffer (1x PBS with 0.05% Tween-20).

4.4) Add 50 µl/well of sample diluent (1% bovine serum albumin (BSA), 0.2% Tween-20 in RPMI-1640), then add 50 µl of sample to appropriate wells. Include at least three wells with sample diluent only as mock/negative controls. Incubate for 2 hr at 37 ^{o}C or overnight at 4 ^{o}C.

4.5) Prepare the primary antibody solution fresh before use. Make a 1:2,000 fold dilution of the primary antibody working stocks using the primary antibody diluent (12% FBS in RPMI-1640). Ensure to prepare enough for the use of 100 µl solution per each sample/control well in a 96-well plate.

4.5.1) For example, to make enough solution for one 96-well plate, add 5 µl of the primary antibody working stocks to the primary antibody diluent for a final volume of 10 ml.

4.6) Wash capture plate 5 times with wash buffer.

4.7) Add 100 µl of the primary antibody solution to each well. Incubate for 1 h at 37 ^{o}C in a 5% CO_2 atmosphere.

4.8) Prepare the secondary antibody solution fresh before use. Make a 1:14,400 fold dilution of the secondary antibody (1 mg/ml Goat anti-rabbit HRP) using the secondary antibody diluent (7% FBS, 0.01% Tween-20 in RPMI-1640). To reduce pipetting errors, perform a two-step serial dilution. Ensure to prepare enough for the use of 100 µl solution per each sample/control well in a 96-well plate.

4.8.1) For example, to make enough solution for one 96-well plate, first add 1 µl of the secondary antibody to 99 µl of the secondary antibody diluent. Then add 70 µl of first dilution to the secondary antibody diluent for a final volume of 10 ml.

4.9) Wash capture plate 5 times with wash buffer.

4.10) Add 100 µl of the secondary antibody solution to each well. Incubate for 1 hr at 37 ^{o}C in a 5% CO_2 atmosphere.

4.11) Wash plate 5 times with wash buffer.

4.12) Add 100 µl of room temperature TMB substrate. Incubate 30 min at room temperature in a closed container to protect from light.

4.13) Add 100 µl of room temperature stop solution (1 N H_2SO_4).

4.14) 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. Consider a well to contain infectious virus if the absorbance value is at least three times higher than the value read from mock / negative control wells. Calculate $TCID_{50}$ of the viral stock using

the Reed-Meunch method[31].

5) **Establish viral growth kinetics**

5.1) Monoinfection

5.1.1) Seed $3x10^5$ PHA-stimulated PBMC/well in 48-well plates in a total volume of 500 µl/well. Keep the culture plates at 37 ^oC in a 5% CO_2 atmosphere until inoculation.

5.1.2) For each virus, prepare an inoculum containing 6,000 IU in 2 ml of cIMDM.

5.1.3) Inoculate wells in triplicate by adding 500 µl of the inoculum (1,500 IU) to the seeded cells. The final volume of the infected cell culture is 1 ml/well and the MOI is 0.005.

5.1.4) Aliquot 200 µl of the remaining inoculum to each of two 96-well plates for RNA isolation, one of which is saved as a backup.

5.1.5) Incubate cultures at 37 ^oC in a 5% CO_2 atmosphere for 16-24 h.

5.1.6) Wash cultures 16-24 h after inoculation.

5.1.6.1) Remove and discard 750 µl of culture supernatant.

5.1.6.2) Add 750 µl of fresh cIMDM. Wrap plates in plastic wrap and spin for 10 minutes at 300 x g. Remove and discard 750 µl supernatant.

5.1.6.3) Add 750 µl of fresh cIMDM. Incubate at 37 ^oC with a 5% CO_2 atmosphere (day 1)**.**

5.1.7) Sample cultures daily from day 2 to day 7.

5.1.7.1) Transfer 500 µl of culture supernatant to a 1.8 ml centrifuge tube. Spin for 1 min at 3000 x g.

5.1.7.2) Transfer 200 µl of the cell-free supernatant to the two 96-well sample plates for RNA isolation, again saving one plate as backup. Store supernatants at -80 ^oC until RNA isolation.

5.1.7.3) Add 500 µl fresh cIMDM to each culture. Incubate at 37 ^oC in a 5% CO_2 atmosphere.

5.1.7.4) Discard cultures into Wescodyne at the end of the experiment.

5.1.7.5) Isolate RNA from 200 µl of supernatant (use commercial kits such as QIAamp Viral RNA Mini Kit) following the manufacturer's standard protocol. For a large

number of samples, use the Qiagen QIAxtractor.

5.1.7.6) Store RNA samples at -80 ^{o}C until cDNA synthesis.

5.2) cDNA synthesis (Reverse transcription)

5.2.1) For each RNA sample, add 1.2 nmol of dNTP and 1.2 pmol of cDNA synthesis primer, (5'-GTTGATCCTTTAGGTATCTTTCCACAGC-3', HXB2 nucleotide 7968 to 7995) to 10 µl of viral RNA. Add water to a final volume of 14 µl. Flick the tube to mix and spin briefly to collect liquid at the bottom of the tube.

5.2.2) Incubate mixture for 5 min at 65 ^{o}C, then hold at 4 ^{o}C until the master mix is prepared.

5.2.3) Prepare master mix using 5x first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 5 mM DTT, 120 U of SuperScriptIII and 240 U of Rnase inhibitor. Add water to final volume of 10 µl.

5.2.4) Add 10 µl of master mix to RNA mixture, flick to mix and spin to collect.

5.2.5) Incubate mixture for 90 min at 50 ^{o}C to allow synthesis of cDNA. Incubate for 15 minutes at 70 ^{o}C to inactivate reverse transcriptase. Hold at 4 ^{o}C as needed.

5.2.6) Add 2 U of Rnase H, flick to mix, and then spin to collect.

5.2.7) Incubate 20 min at 37 ^{o}C. Store cDNA at -20 ^{o}C.

5.3) cDNA quantitation using qPCR system

5.3.1) Prepare a standard dilution series. Do 10-fold serial dilution, from 3 x 10^6 copies/µl down to 30 copies/µl, of pNL4-3VifA. Use distilled water for dilutions. Prepare the standard dilution series fresh before use or prepare small batches and keep at -20 ^{o}C. Do not freeze-thaw the standards more than three times.

5.3.2) Set up a 96-well qPCR reaction plate. Ensure that each plate contains at least one well of negative controls, a triplicate of the standard dilution series, and at least a duplicate of each cDNA sample.

5.3.3) For each qPCR reaction, use 12.5 µl of qPCR master mix, 0.2 µM probe, 0.8 µM each of the forward and reverse primers and 1 µl of cDNA or the standard dilution series or water/buffer (for the negative control well). Add water to a final volume of 25 µl. The qPCR probe is light sensitive. Keep it in closed container.

5.3.3.1) To detect cDNA derived from pNL4-3VifA based molecular clone, use the VifA primer-probe: VifAB forward primer (GGTCTGCATACAGGAGAAAGAGACT), VifA reverse primer (5'-AGGGTCTACTTGTGTGCTATATCTCTTTT-3') and VifAB probe

(6FAM-5'-CTCCATTCTATGGAGACTC-3'-MGBNFQ). For cDNA derived from pNL4-3VifB based clone, use the VifB primer-probe: VifAB forward primer, VifAB probe and VifB reverse primer (5'-CACCTGCGTGCTATACCTTTTCT-3').

5.3.4) Set PCR cycling parameters to 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, and 40 cycles of 95 ^{o}C for 15 sec and 60 ^{o}C for 1 min. Consult the manufacturer's support document for operation of the qPCR machine.

5.3.5) Calculate a standard curve using data from the triplicate standard dilution series. Compare amplification data of the cDNA sample to the standard curve to determine the copy number. Consult the manufacturer's support document for data processing.

5.4) Determine viral exponential growth phase

5.4.1) Plot viral growth kinetics with the sampling day along the X-axis and the cDNA copy number along the Y-axis and identify the viral exponential growth phase, i.e., when viral cDNA copy numbers increase in an exponential progression.

5.4.2) Use the GRC web tool (http://indra.mullins.microbiol.washington.edu/grc/) to calculate viral growth rate (*g*). In this application, the GRC tool accepts cDNA copy numbers from at least two time points as input, and outputs the viral growth rate (*g*). Use only time point data within the exponential growth phase (see step 5.4.1 above) to obtain accurate growth rates. For a detailed description of the mathematical model used in the GRC web tool, see[20].

6) Growth Competition Assay

6.1) Seed 3 x 10^5 PHA-stimulated PBMCs (or 1 x 10^5 CEMx174 cells) in 500 µl total volume per well in a 48 well flat-bottomed plate.

6.2) Keep plate in 37 ^{o}C in a 5% CO_2 atmosphere until inoculation.

6.3) For each virus, prepare 3 ml of inoculum containing 6,000 IU.

6.4) Transfer 1.5 ml of each viral inoculum to a sterile tube to create the dual infection inoculum. Add 500 µl of the dual inoculum (1,500 IU) to 3 x 10^5 cells in a 48-well plate. The final culture volume is 1 ml/well. Aliquot 200 µl of the inoculum to two 96-well plates for RNA isolation; save one plate as a back up.

6.5) Incubate inoculated cells at 37 ^{o}C with a 5% CO_2 atmosphere for 16-24 hr.

6.6) Wash cultures 16-24 h after inoculation.

6.6.1) Remove and discard 750 µl of culture supernatant.

6.6.2) Add 750 µl of fresh cIMDM. Wrap plates in plastic wrap and spin for 10 minutes at 300 x g. Remove and discard 750 µl supernatant.

6.6.3) Add 750 µl of fresh cIMDM. Incubate at 37 ^{o}C with a 5% CO_2 atmosphere (day 1).

6.7) Select sampling times to include at least 3 time points within the exponential growth phase observed in step 5.4.1.

6.7.1) For each sampling, follow step 5.1.7.

6.8) Perform cDNA synthesis as described in section 5.2.

6.9) Determine the viral variant ratio using qPCR.

6.9.1) Prepare a standard serial dilution series in triplicate, diluting 10-fold in each step from 3 x 10^6 copies/µl to 30 copies/µl of pNL4-3VifA.

6.9.2) Set up a 96-well qPCR reaction plate. Ensure that each plate contains at least one negative control, the standard dilution series in triplicate, and duplicates of each cDNA sample.

6.9.3) For each qPCR reaction, use 12.5 µl of qPCR Master Mix, 0.2 µM probe, 0.8 µM each of the forward and reverse primers and 1 µl of cDNA or the standard dilution series or water/buffer (for the negative control wells). Add water to a final volume of 25 µl. The qPCR probe is light sensitive, keep it in closed container.

6.9.3.1) Use the VifA primer-probe to detect signals in the negative controls and the standard dilution series and with one duplicate of the cDNA sample. Use the VifB primer-probe with the other duplicate of the cDNA sample.

6.9.4) Set PCR cycling parameters to 50 ^{o}C for 2 min, then 95 ^{o}C for 10 min, and then 40 cycles of 95 ^{o}C for 15 sec and 60 ^{o}C for 1 min. Consult the manufacturer's support document for operation of the qPCR machine.

6.9.5) Calculate the standard curve using amplification data from the standard dilution series. Compare amplification data of the cDNA samples to the standard curve to determine copy number. Consult the manufacturer's support document for data processing.

6.9.6) Use the GRC web tool (http://indra.mullins.microbiol.washington.edu/grc/) to calculate relative viral fitness (*d*).

Note: The GRC tool accepts cDNA copy numbers or chromatogram peak heights from at least two time points as the input, and outputs the net growth rate difference (*d*) between the two viruses. While the tool can calculate the net growth rate from two time

point data, it is strongly recommended to input data from three or more time points. Use only data obtained from time points within the exponential growth phase (see step 5.4) to obtain accurate growth rates. For a detailed description of the mathematical model used in the GRC web tool, see[20].

6.10) Determine viral ratios using chromatogram peak-heights

6.10.1) PCR amplify HIV-1 *vif* fragments containing the VifAB sequence tag using VifFwd (5′-GAAAGAGACTGGCATTTGGGTCAGGG-3′; HXB2 positions 5266–5291) and VifRev primers (5′-GTCTTCTGGGGCTTGTTCCATCTGTCC-3′; HXB2 positions 5579–5553).

6.10.1.1) For each PCR reaction, use 1 µl of cDNA, 1X NH_4 buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U of Taq Polymerase, and 0.45 µM of each primer. Add water to a final volume of 50 µl.

6.10.1.2) Set PCR cycling parameters to 3 cycles at 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, and 70 ^{o}C for 1 min, followed by 34 cycles at 94 ^{o}C for 15 sec, 58 ^{o}C for 30 sec, and 70 ^{o}C for 1 min, and then hold at 4 ^{o}C.

6.10.2) Purify PCR products using the QIAquick PCR purification kit according to the manufacturer's protocol.

6.10.3) Submit purified PCR products to a DNA sequencing service provider for Sanger sequencing.

6.10.4) Check the average read quality score, which should be provided by the sequencing service. If the average base call accuracy is less than 85%, redo step 6.10.1 to 6.10.3

6.10.5) Use the ChromatQuan web tool (http://indra.mullins.microbiol.washington.edu/cgi-bin/chromatquant.cgi) to calculate viral ratio at each time point. The tool requires the sequence trace file (*.ab1) and the sequence 5' to the nucleotide site of interest. The tool measures the peak intensity at the specified site. The ratio of the peak intensity corresponds to the ration of the two viruses **(Figure 4B).**

6.10.6) Use the GRC web tool (http://indra.mullins.microbiol.washington.edu/grc/) and the recorded peak intensities as the input to calculate viral relative fitness (*d*). See step 6.10.6.[20]

REPRESENTATIVE RESULTS:

To study the fitness impact of single amino acid changes in HIV-1 Gag-p24, we used oligonucleotide directed mutagenesis to introduce mutations into a pNL4-3 plasmid containing the HIV-1 COTB-p24 gene[23,32,33]. Viral stocks were generated by transfection of 293T cells and harvested after 48 hours. We estimated the 50% tissue culture

infectious dose ($TCID_{50}$) of each viral stock by the Reed-Muench method[31]. The $TCID_{50}$ of the prototype and mutant viruses ranged from 10^4 to 10^5 IU/ml (**Figure 3A**).

The growth kinetics of the recombinant viruses were established in PBMCs from a single donor at an MOI of 0.005. RT-qPCR was used to measure viral cDNA copy number daily for six days. All mutant and prototype viruses grew exponentially between day 2 and day 4, following which viral growth slowed, as indicated by a decreased slope of the viral RNA copy number increase (**Figure 3B**). 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). In the exponential growth phase, all three mutants had a slower growth rate (*g*) than the prototype virus (**Figure 3C**).

All three mutants were competed against the prototype virus in growth competition assays at a total MOI of 0.005. Viral growth kinetics in dually infected cultures were similar to that of monoinfection; the viral exponential growth phase was between days 2 and 4, and viral growth reached a plateau around day 5 (**Figure 4A**).

Viral growth rate differences were derived from the change in the viral ratio over time. The viral ratio was calculated based on cDNA copy number of the reference and the mutant viruses using RT-qPCR, and by comparing peak heights in sequence chromatograms at nucleotide sites distinguishing the two viruses (**Figure 4B)**. The growth rate differences determined using the peak-height and viral RNA copy number methods yielded similar results (**Figure 4C**). All three mutants had lower replication fitness than the prototype viruses with the mutant I256V having the lowest fitness (**Figure 4C**).

Figure 1. Flow diagram of the protocols presented in this paper. The Virus Preparation protocols concern construction of HIV-1 recombinant clones and generation of viral stocks. The Fitness Assays protocols are for establishing viral growth kinetics and determining relative viral fitness. Dashed lines represent alternative flows for the protocols. For example, a mutation can be introduced directly into HIV-1 NL4-3 molecular clone without generating a recombinant clone.

Figure 2. Construction of HIV-1 NL4-3 COTB Gag-p24 recombinant molecular clones using overlap extension PCR. A) Design the chimeric primers. The 5' halves of the primers contain the NL4-3 vector sequence and the 3' halves contain the ends of the insert sequence. B) Chimeric primers are used to amplify the insert fragment, COTB Gag-p24. C) The insert fragments are used as primers for a second PCR to generate a new recombinant plasmid.

Figure 3. Viral growth characteristics in PBMCs. A) Log_{10} $TCID_{50}$ of viral stocks. B) Viral growth kinetics in monoinfections begun at an MOI = 0.005. C) Viral growth rates over six days, including the exponential growth phase (days 2-4) derived from panel B. The values shown represent the average of three replicates from one experiment. The error bars represent 95% confidence intervals.

Figure 4. Viral growth and relative fitness determinations. A) Viral growth in dually infected PBMC cultures. B) Ratios of T242N and prototype viruses determined using RT-qPCR or the sequencing peak-height method. C) Net viral growth rate differences (d) between the mutant and the prototype viruses in dually infected cultures, as shown in A. d values were calculated from the viral ratio data shown in B. The values shown represent the average of three replicates from one experiment. The error bars represent 95% confidence intervals.

DISCUSSION:

The protocols presented consisted of two main parts: construction of recombinant HIV-1 molecular clones and growth competition assays. In order to distinguish two viruses in a dually infected cell culture, it is important that the competing molecular clones contain sequence tags, which can be detected by a RT-qPCR primer-probe assay or by Sanger sequencing. This protocol makes use of the VifA and VifB tags, which occupy the same region of HIV-1 NL4-3 *vif* and encode the same amino acid sequence but differ by six synonymous mutations. These mutations were shown not to affect viral replication fitness[20]. The PCR-based cloning method used in this protocol provides more flexibility in selecting cloning sites, compared to restriction site based cloning. However, the efficiency of PCR cloning decreases as the insert size increases. The current limit of the insert size is ~5 kb[34]. 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.

At least three sampling time points should be examined within the exponential growth phase[9]. The viral growth kinetics must first be established using daily sampling to determine the appropriate culture period and sampling time points for the growth competition. One factor that affects viral growth kinetics is the multiplicity of infection (MOI). This protocol uses a total MOI of 0.005 for both monoinfection and growth competition, as it was shown to yield more robust results than lower MOIs[9]. Nonetheless, lower MOIs can be used to obtain a longer exponential growth phase if necessary, but at the expense of result consistency. 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 are 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 excess[9].

The protocol for determining the $TCID_{50}$, 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 cells[35]. 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.

Two different methods to determine the viral ratio for calculating fitness are included in the protocol. The first uses RT-qPCR to measure viral cDNA copy number at each sampling time point. Viral replication fitness was then calculated from the viral ratio, based on cDNA copy number. Alternatively, the viral ratio can be determined based on the ratio of chromatogram peak height at the VifAB tag sites. The two methods yielded comparable results (**Figure 4).** The chromatogram peak height method can be applied to other HIV-1 strains without an engineered sequence tag. For RT-qPCR, the use of primers or probes to distinguish viral variants must first be carefully evaluated (see[20]). Nevertheless, RT-qPCR provides better sensitivity for samples with a small amount of viral RNA, such as those from the first time point within the exponential growth phase. Direct measurement of viral cDNA also allows detection of technical problems that may arise from the RNA extraction and cDNA synthesis. Using molecular clones with sequence tags provides a cost effective solution to the RT-qPCR methods, as only two pairs of primers and probes are needed to study multiple viruses. This strategy also avoids the problem of peak-height variation in sequence chromatograms due to neighboring bases[37], as sequencing is done at the same site across all viruses in the study. 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].

ACKNOWLEDGMENTS:
These studies were funded by US Public Health Services grants P01AI057005, R01AI047734, R01AI111806 and the Functional Profiling and Computational Biology Core of the University of Washington's Center for AIDS Research (P30 AI027757).

DISCLOSURES:
The authors declare that they have no competing financial interests.

REFERENCES:

1 Domingo, E. & Holland, J.J. RNA virus mutations and fitness for survival. *Annu Rev Microbiol* **51,** 151-178, doi: 10.1146/annurev.micro.51.1.151 (1997).
2 Domingo, E. Mechanisms of viral emergence. *Veterinary research* **41** (6), 38, doi: 10.1051/vetres/2010010 (2010).

3 Martinez-Picado, J. & Martinez, M.A. HIV-1 reverse transcriptase inhibitor resistance mutations and fitness: a view from the clinic and ex vivo. *Virus Res* **134** (1-2), 104-123, doi: 10.1016/j.virusres.2007.12.021 (2008).
4 Troyer, R.M. *et al.* Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLoS Pathog* **5** (4), e1000365, doi: 10.1371/journal.ppat.1000365 (2009).
5 Rolland, M. *et al.* Amino-acid co-variation in HIV-1 Gag subtype C: HLA-mediated selection pressure and compensatory dynamics. *PLoS One* **5** (9), e12463, doi: 10.1371/journal.pone.0012463 (2010).
6 Abraha, A. *et al.* CCR5- and CXCR4-tropic subtype C human immunodeficiency virus type 1 isolates have a lower level of pathogenic fitness than other dominant group M subtypes: implications for the epidemic. *J Virol* **83** (11), 5592-5605, doi: 10.1128/jvi.02051-08 (2009).
7 Quinones-Mateu, M.E. *et al.* A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. *J Virol* **74** (19), 9222-9233 (2000).
8 Ball, S.C. *et al.* Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C. *J Virol* **77** (2), 1021-1038, doi: 10.1128/JVI.77.2.1021-1038.2003 (2003).
9 Lanxon-Cookson, E.C. *et al.* Factors affecting relative fitness measurements in pairwise competition assays of human immunodeficiency viruses. *J Virol Methods* **194** (1-2), 7-13, doi: 10.1016/j.jviromet.2013.07.062 (2013).
10 Garcia-Lerma, J.G., MacInnes, H., Bennett, D., Weinstock, H. & Heneine, W. Transmitted human immunodeficiency virus type 1 carrying the D67N or K219Q/E mutation evolves rapidly to zidovudine resistance in vitro and shows a high replicative fitness in the presence of zidovudine. *J Virol* **78** (14), 7545-7552, doi: 10.1128/jvi.78.14.7545-7552.2004 (2004).
11 Sharma, P.L. & Crumpacker, C.S. Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation. *J Virol* **71** (11), 8846-8851 (1997).
12 Martinez-Picado, J., Savara, A.V., Sutton, L. & D'Aquila, R.T. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J Virol* **73** (5), 3744-3752 (1999).
13 Wang, J. *et al.* The HIV-1 reverse transcriptase mutants G190S and G190A, which confer resistance to non-nucleoside reverse transcriptase inhibitors, demonstrate reductions in RNase H activity and DNA synthesis from tRNA(Lys, 3) that correlate with reductions in replication efficiency. *Virology* **348** (2), 462-474, doi: 10.1016/j.virol.2006.01.014 (2006).
14 Song, H. *et al.* Impact of immune escape mutations on HIV-1 fitness in the context of the cognate transmitted/founder genome. *Retrovirology* **9** (89), doi: 10.1186/1742-4690-9-89 (2012).
15 Ali, A. & Yang, O. A novel small reporter gene and HIV-1 fitness assay. *Journal of Virological Methods* **133** (1), 41-47, doi: 10.1016/j.jviromet.2005.10.016 (2006).
16 van Maarseveen, N.M. *et al.* A novel real-time PCR assay to determine relative replication capacity for HIV-1 protease variants and/or reverse transcriptase variants. *J Virol Methods* **133** (2), 185-194, doi: 10.1016/j.jviromet.2005.11.008 (2006).

17 Liu, Y. *et al.* Evolution of human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitopes: fitness-balanced escape. *J Virol* **81** (22), 12179-12188, doi: 10.1128/jvi.01277-07 (2007).
18 Anastassopoulou, C.G., Ketas, T.J., Klasse, P.J. & Moore, J.P. Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41. *Proc Natl Acad Sci U S A* **106** (13), 5318-5323, doi: 10.1073/pnas.0811713106 (2009).
19 Abraha, A., Troyer, R.M., Quinones-Mateu, M.E. & Arts, E.J. Methods to determine HIV-1 ex vivo fitness. *Methods Mol Biol* **304,** 355-368, doi: 10.1385/1-59259-907-9:355 (2005).
20 Liu, Y. *et al.* A sensitive real-time PCR based assay to estimate the impact of amino acid substitutions on the competitive replication fitness of human immunodeficiency virus type 1 in cell culture. *J Virol Methods* **189** (1), 157-166, doi: 10.1016/j.jviromet.2012.10.016 (2013).
21 Brumme, Z.L. *et al.* Reduced replication capacity of NL4-3 recombinant viruses encoding reverse transcriptase-integrase sequences from HIV-1 elite controllers. *J Acquir Immune Defic Syndr* **56** (2), 100-108, doi: 10.1097/QAI.0b013e3181fe9450 (2011).
22 Nomura, S. *et al.* Significant reductions in Gag-protease-mediated HIV-1 replication capacity during the course of the epidemic in Japan. *J Virol* **87** (3), 1465-1476, doi: 10.1128/jvi.02122-12 (2013).
23 Rolland, M. *et al.* HIV-1 conserved-element vaccines: relationship between sequence conservation and replicative capacity. *J Virol* **87** (10), 5461-5467, doi: 10.1128/jvi.03033-12 (2013).
24 Martinez-Picado, J. *et al.* Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* **80** (7), 3617-3623, doi: 10.1128/jvi.80.7.3617-3623.2006 (2006).
25 Brockman, M.A. *et al.* Escape and Compensation from Early HLA-B57-Mediated Cytotoxic T-Lymphocyte Pressure on Human Immunodeficiency Virus Type 1 Gag Alter Capsid Interactions with Cyclophilin A. *J Virol* **81** (22), 12608-12618 (2007).
26 Miura, T. *et al.* HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying gag-protease from elite controllers of human immunodeficiency virus type 1. *J Virol* **83** (1), 140-149, doi: 10.1128/jvi.01471-08 (2009).
27 JoVE Science Education Database, Cambridge, MA, Basic Methods in Cellular and Molecular Biology, PCR: The Polymerase Chain Reaction, doi: 10.3791/5056 (2014)
28 Lee, P.Y., Costumbrado, J., Hsu, C.Y. & Kim, Y.H. Agarose gel electrophoresis for the separation of DNA fragments. *Journal of visualized experiments : JoVE* **62** (Apr 20), doi: 10.3791/3923 (2012).
29 JoVE Science Education Database, Cambridge, MA, Basic Methods in Cellular and Molecular Biology, Restriction Enzyme Digests, doi: 10.3791/5070 (2014)
30 McClure, J., van't Wout, A.B., Tran, T. & Mittler, J.E. Granulocyte-monocyte colony-stimulating factor upregulates HIV-1 replication in monocyte-derived macrophages cultured at low density. *J Acquir Immune Defic Syndr* **44** (3), 254-261, doi: 10.1097/QAI.0b013e318030f5c5 (2007).
31 Reed, L.J. & Muench, H. A simple method of estimating fifty percent endpoints. *Am J Hyg.* **27** (3), 493-497 (1938).

32 Nickle, D.C. *et al.* Consensus and ancestral state HIV vaccines. *Science* **299** (5612), 1515-1518 (2003).
33 Rolland, M. *et al.* Reconstruction and function of ancestral center-of-tree human immunodeficiency virus type 1 proteins. *J Virol* **81** (16), 8507-8514, doi: 10.1128/jvi.02683-06 (2007).
34 Bryksin, A.V. & Matsumura, I. Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *Biotechniques* **48** (6), 463-465, doi: 10.2144/000113418 (2010).
35 Spira, A.I. & Ho, D.D. Effect of different donor cells on human immunodeficiency virus type 1 replication and selection in vitro. *J. Virol.* **69** (1), 422-429 (1995).
36 Manocheewa, S., Swain, J.V., Lanxon-Cookson, E., Rolland, M. & Mullins, J.I. Fitness costs of mutations at the HIV-1 capsid hexamerization interface. *PLoS One* **8** (6), e66065, doi: 10.1371/journal.pone.0066065 (2013).
37 Zakeri, H., Amparo, G., Chen, S.M., Spurgeon, S. & Kwok, P.Y. Peak height pattern in dichloro-rhodamine and energy transfer dye terminator sequencing. *Biotechniques* **25** (3), 406-410, 412-404 (1998).
38 Lalonde, M.S. *et al.* Sensitive oligonucleotide ligation assay for low-level detection of nevirapine resistance mutations in human immunodeficiency virus type 1 quasispecies. *J Clin Microbiol* **45** (8), 2604-2615, doi: 10.1128/JCM.00431-07 (2007).

Figure 1

Figure 1

Click here to download Figure: JoVE_figures_102114_fig1.pdf

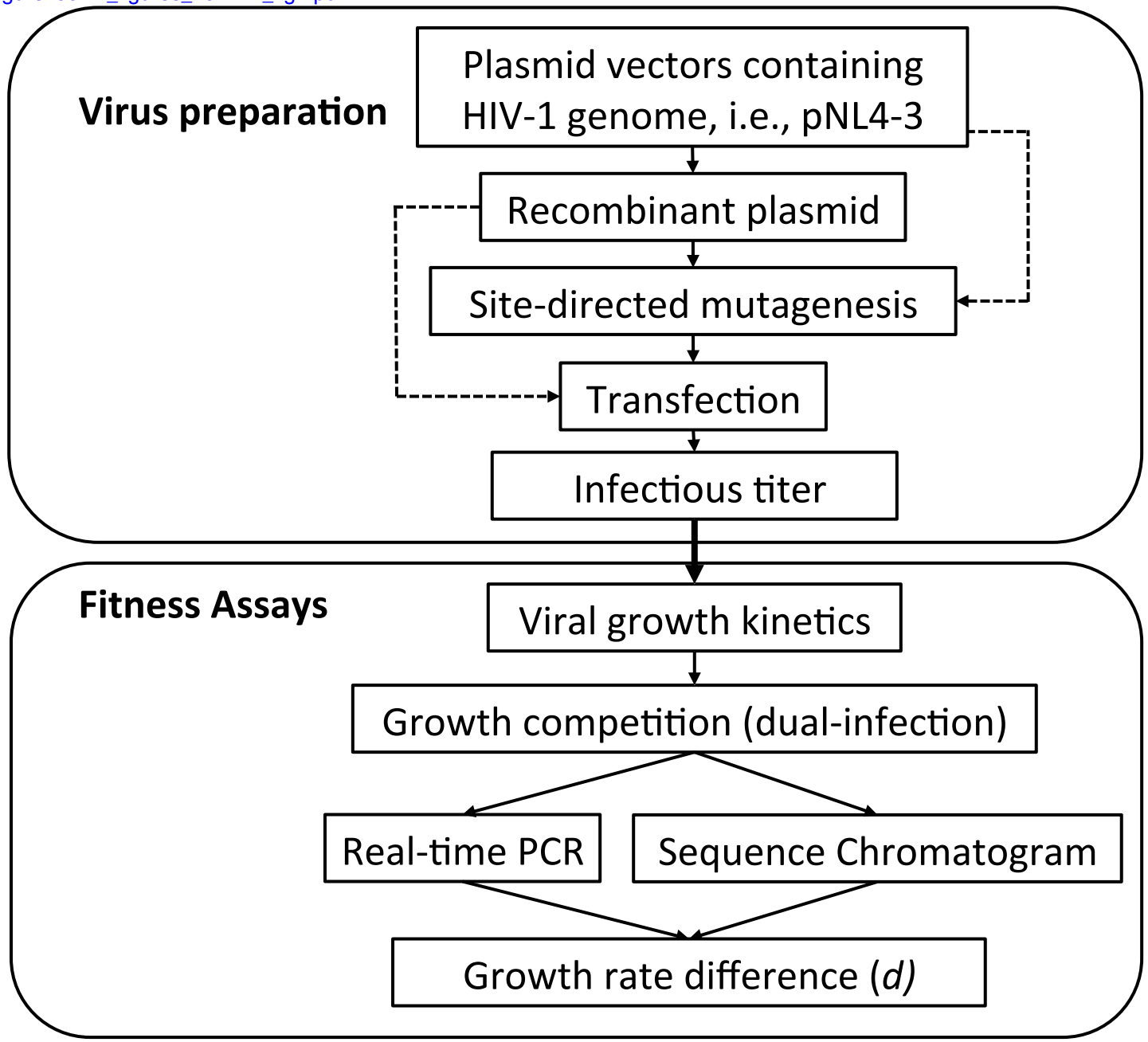


Figure 2

Figure 2

Click here to download Figure: JoVE_figures_102114_fig2.pdf

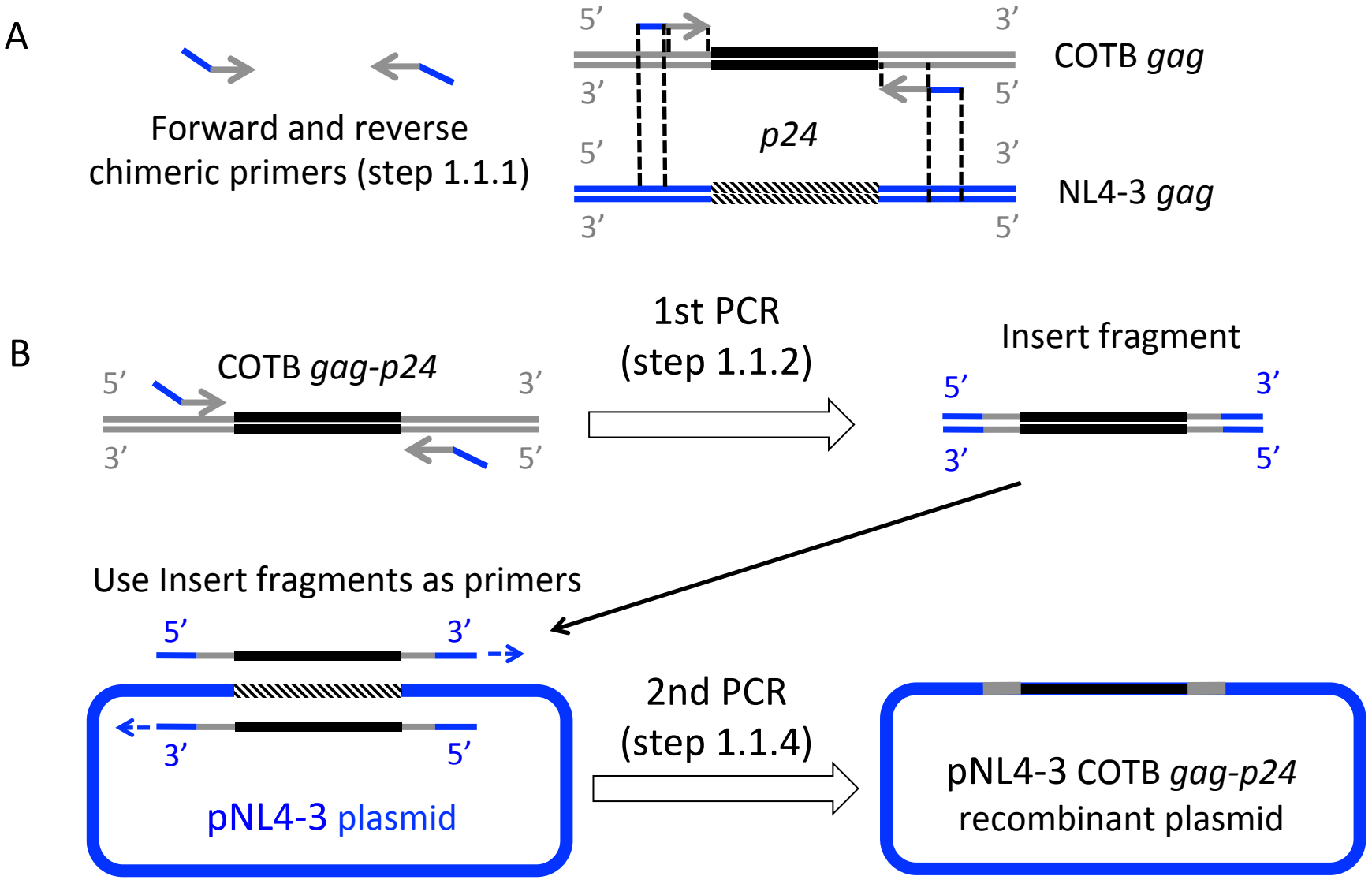
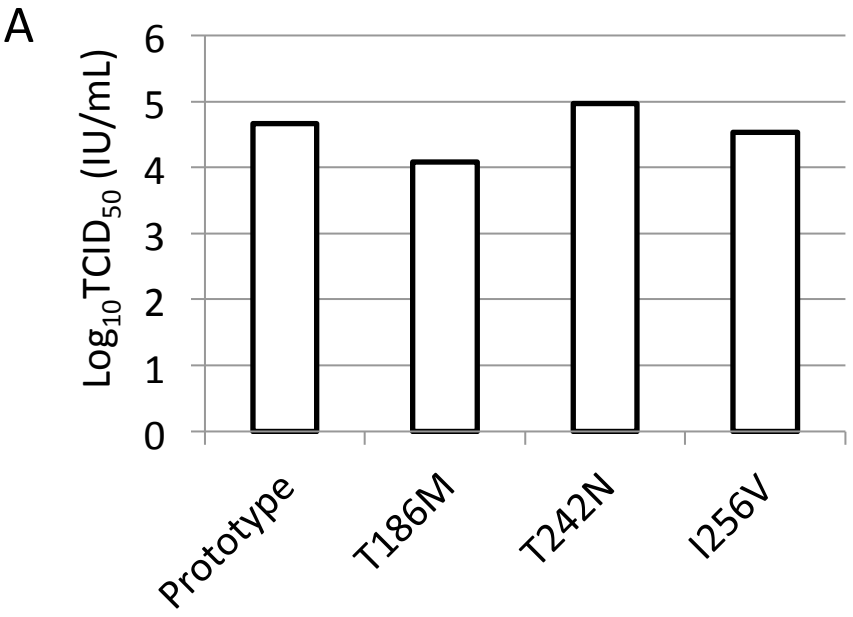
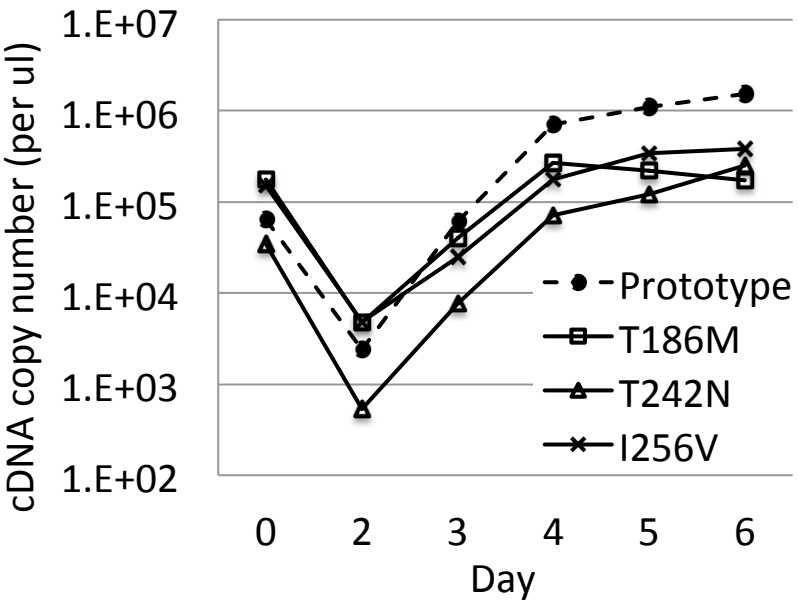


Figure 3

Figure 3





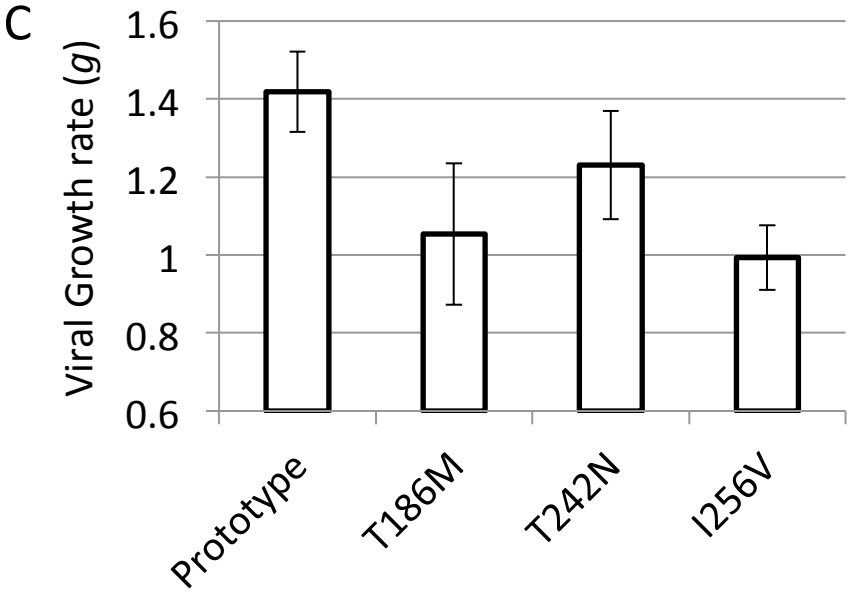
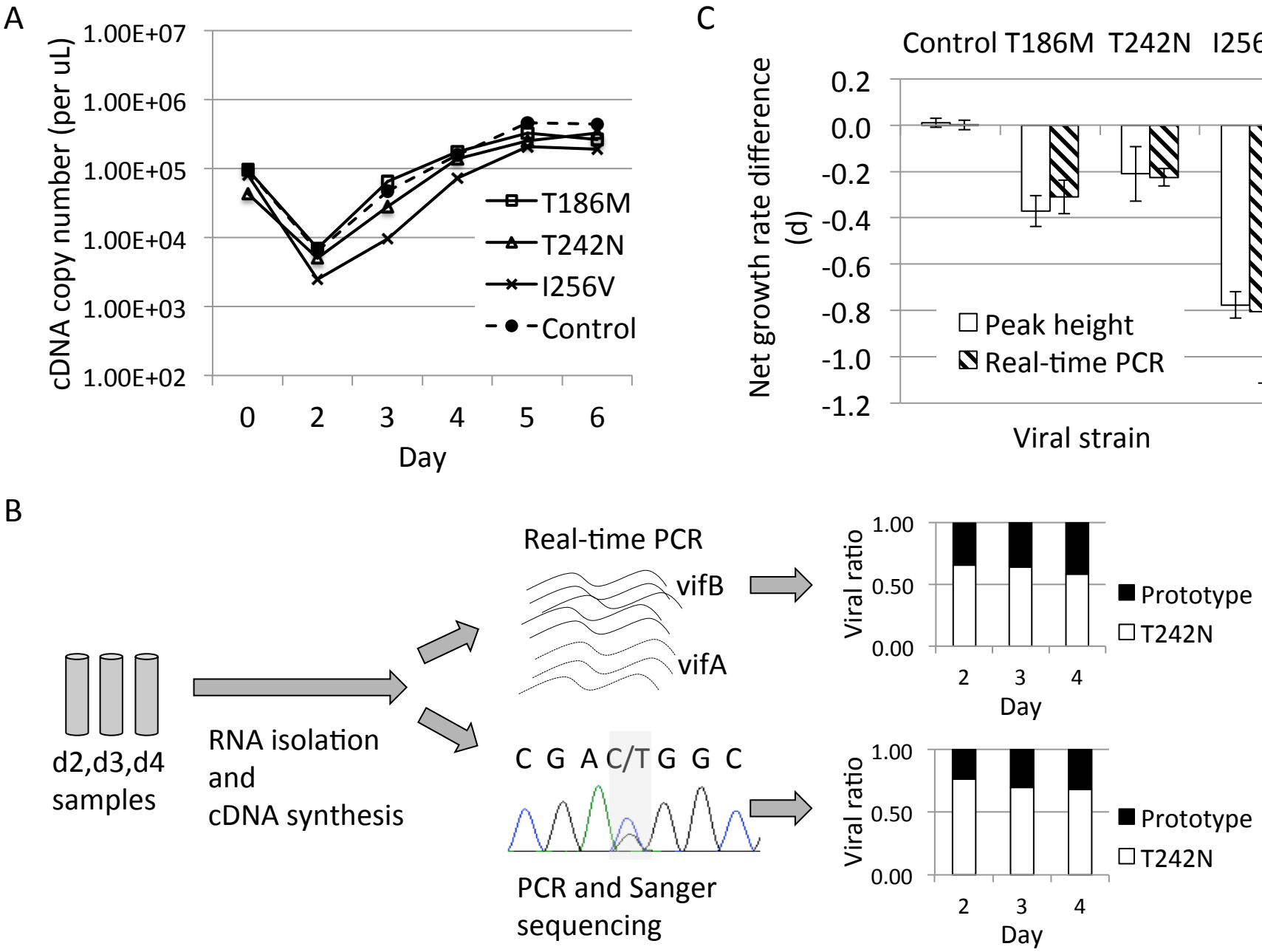


Figure 4

Figure 4

Click here to download Figure: JoVE_figures_102114_fig4.pdf



Excel Spreadsheet- Table of Materials/Equipment
Click here to download Excel Spreadsheet- Table of Materials/Equipment: JoVE_Materials_8Oct2014.xlsx

Step	Name of Material/ Equipment
Construction of recombinant clones	Chimeric primer/Mutagenic/Sequencing primers
	pNL4-3VifA and pNL4-3VifB plasmid
	High-Fidelity DNA polymerase
	High-fidelity buffer
	dNTP
	Thermal cycler
	DNA loading dye
	1kb Plus DNA ladder
	QIAquick PCR Purification Kit
	QIAquick gel extraction kit
	D pnI enzyme
	TOP10 chemically competent *Escherichia coli*
	Luria Broth base powder
	Carbenicillin
	QIAprep Spin Miniprep kit
	EndoFree Plasmid Maxi Kit
Transfection	X-tremeGENE 9 DNA transfection reagent
	HEK 293T-17
	0.22um filter top tube
Cell culture	Dulbecco's Modified Eagle Medium (DMEM)
	Iscove′s Modified Dulbecco′s Medium (IMDM)
	RPMI-1640 media
	Phytohemagglutinin (PHA)
	Human Interleukin-2
	Fetal bovine serum
	Penicillin and Streptomycin
	6 well plate
	48 well plate
	96 well flat-bottomed plate
	96 well round-bottomed plate
	1.5 ml Microcentrifuge Tube
	1.5 mL tubes w/ O-ring
	50 mL conical tube
ELISA	Triton-X 100
	Phosphate buffer saline (PBS)
	Fetal bovine serum (FBS)
	glycerol
	Tween 20
	Bovine Serum Albumin (BSA)
	Rabbit anti-HIV-1 SF2 p24 antiserum
	Goat anti-rabbit HRP
	TMB Microwell Peroxidase Substrate

	H_2SO_4
	HIV p24 standard
	Microplate reader
RNA isolation	QIAxtractor
cDNA synthesis	QIAamp Viral RNA Mini Kit
	dNTP
	SuperscriptIII
	First-strand buffer
	Dithiothreitol (DTT)
	Rnase inhibitor
	RnaseH
qPCR	Real-time qPCR machine
	TaqMan® Gene Expression Master Mix
	Forward, reverse primer
	Probe
	optical 96 well reaction plate
PCR+sequencing	Taq DNA polymerase (Biolase)
	NH4 buffer
	$MgCl_2$
	Sequencing primer
	QIAxcel
	DNA sequencing service provider
	GRC web tool
	ChromatQuan web tool

Company	Catalog Number
IDT	N/A
N/A	N/A
Thermo Scientific	F-549S
Thermo Scientific	F-549S
Bioline	Bio-39026
Life Technologies	N/A
Thermo Scientific	R0631
Invitrogen	10787-018
Qiagen	28104
Qiagen	28704
New England Biolabs	R0176S
Invitrogen	C4040-10
Invitrogen	12795-084
Research Products International	C46000-25.0
Qiagen	27104
Qiagen	12362
Roche	6365787001
ATCC	CRL-11268
VWR International	89220-716
Life Technologies	10566016
Life Technologies	31980-030
Life Technologies	61870-036
Thermo Scientific	R30852801
Roche	11147528001
JR Scientific	43640
Corning Cellgro	30-001-CI
VWR Scientific	73520-906
VWR Scientific	62407-338
ISC Bioexpress	T-3015-4
BD Falcon	353077
Mt. Baker Bio	MBD-1500
VWR Scientific	89004-290
ISC Bioexpress	C-3317-6
Sigma-Aldrich	X100
Invitrogen	14190-250
Sigma-Aldrich	F0392
Sigma-Aldrich	G5516
Sigma-Aldrich	P1379
Sigma-Aldrich	A2153
NIH AIDS Reagent Program	4250
KPL	474-1516
KPL	52-00-01

Fisher Scientific	A300-500
AIDS and Cancer Virus program (NCI-Frederick)	N/A
Molecular Devices	N/A
Qiagen	N/A
Qiagen	52906
Bioline	Bio-39026
Invitrogen	18080-085
Invitrogen	18080-085
Invitrogen	18080-085
Roche	3335402001
Invitrogen	18021-071
Life Technologies	N/A
Life Technologies	4369016
IDT	N/A
Life Technologies	N/A
Life Technologies	I19N3Q216
Bioline	Bio-21043
Bioline	Bio-21043
Bioline	Bio-21043
IDT	N/A
Qiagen	
http://www.htseq.org/	

http://indra.mullins.microbiol.washington.edu/grc/
http://indra.mullins.microbiol.washington.edu/cgi-bin/chromatquant.cgi

Comments/Description
Custom DNA oligos
Please contact Dr. James I Mullins (jmullins@uw.edu)

Applied Biosystems® GeneAmp® PCR System 9700

http://www.atcc.org/

Causes severe burns by all exposure routes. Use personal protective equipment. Use onl

For order details please contact Julian Bess, Jr. (bessjw@mail.nih.gov); http://ncifrederick

VMax Kinetic ELISA Microplate Reader

http://www.qiagen.com/products/catalog/automated-solutions/sample-prep/qiaxtractor

Applied Biosystems 7300 Real-Time PCR

Custom order

Custom order

Custom order

http://www.qiagen.com/products/catalog/automated-solutions/detection-and-analysis/qiax

y under a chemical fume hood. Wash off immediately with plenty of water for at least 15 min
t.cancer.gov/Programs/Science/Acvp/bio/Bess.aspx

cel-advanced-system

utes.

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Manuscript #:

Title of Article: Pairwise Growth Competition Assay for Determining the Replication Fitness of Human Immunodeficiency Viruses

Author(s): Siriphan Manocheewa, Erinn C. Lanxon-Cookson, Yi Liu, J. Victor Swain, Ushnal Rao, Brandon Maust, Justine E. Sunshine, Moon Kim, Morgane Rolland, James I Mullins

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ■ Open Access

Item 2 (check one box):

■ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the

Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each

such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	James I Mullins
Department:	Microbiology
Institution:	University of Washington
Article Title:	Pairwise Growth Competition Assay for Determining the Replication Fitness of Human Immunodeficiency Viruses
Signature:	Digitally signed by James I Mullins DN: cn=James I Mullins, o=University of Washington, ou, email=jmullins@uw.edu, c=US Date: 2014.08.11 20:03:20 -07'00'
Date:	

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Rebuttal Comments
Click here to download Rebuttal Comments: ResponseToReviewers4.docx

From: "Nam Nguyen" <em@editorialmanager.com>
Subject: Your JoVE Submission JoVE52610R1
Date: October 6, 2014 at 1:35:22 PM PDT
To: "James I Mullins" <jmullins@u.washington.edu>
Reply-To: "Nam Nguyen" <nam.nguyen@jove.com>

CC: aleksandra.jachtorowicz@jove.com

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 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
1 Alewife Center, Suite 200, Cambridge, MA 02140
tel: 617-674-1888

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 $TCID_{50}$, 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 cells[35]. 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 excess[9]."

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 10^4 to 10^6 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 10^6 IU/ml or lower than 10^4 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% CO_2 atmosphere for 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 $TCID_{50}$ of the viral stock using the Reed-Meunch method[31]."

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 excess[9]."

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.